学位論文

Effects of High Temperature Stress on Insect Cell Cycle

（高温ストレスが昆虫の細胞周期に及ぼす影響）

平成 19 年 12 月博士（生命科学）申請

東京大学大学院新領域創成科学研究科

先端生命科学専攻

木内　隆史

Effects of High Temperature Stress on Insect Cell Cycle

Takashi Kiuchi

2007

Department of Integrated Biosciences

Graduate School of Frontier Sciences

The University of Tokyo

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 5

ABBREVIATIONS 6

SUMMARY 9

GENERAL INTRODUCTION 12

CHAPTER 1 17

High Temperature Causes Arrest of Cell Cycle in G_2 Phase in BmN Cells Derived from the Silkworm, *Bombyx mori*

INTRODUCTION 17

MATERIALS AND METHODS 19

RESULTS 23

DISCUSSION 28

FIGURES AND TABLES 32

CHAPTER 2 40

High Temperature Induces Accumulation of ROS and G_2 Cell Cycle Arrest through Activation of p38 MAPK in Insect Cell Line

INTRODUCTION 40

MATERIALS AND METHODS 43

RESULTS 48

DISCUSSION 53

FIGURES AND TABLES 60

CHAPTER 3 73

Effects of High Temperature on the Hemocyte Cell Cycle in Silkworm Larvae

INTRODUCTION 73

MATERIALS AND METHODS 76

RESULTS 81

DISCUSSION 87

FIGURES AND TABLES 92

GENERAL DISCUSSION 99

SUPPLEMENTAL FIGURES AND TABLES 107

CONCLUSIONS 125

REFERENCES 127

ACKNOWLEDGEMENTS

I appreciate Professor Masao Nagata and Associate Professor Fugaku Aoki for their warm encouragement, excellent advice, and valuable discussion through the course of this study.

I am grateful to Professor Haruhiko Fujiwara, Professor Hiroshi Kataoka, Professor Hiroaki Noda, and other faculty members of Department of Integrated Biosciences for reviewing this article.

I wish to thank Professor Shigeyuki Kawano for the provision of laboratory facilities.

I acknowledge Associate Professor Susumu Katsuma at Graduate School of Agricultural and Life Sciences for useful information and suggestion.

I am grateful to Dr. Shigeo Imanishi at National Institute of Agrobiological Sciences for supplying insect cell lines.

I thank my laboratory mates for supporting this study and providing me with wonderful time.

I wish to express our sincere thanks to my family and friends for supporting my student life.

Finally, I pray for the repose of the souls of silkworms used in this study.

ABBREVIATIONS

AA: ascorbic acid

ATM: ataxia telangiectasia mutated

ATR: ATM and Rad3-related

BmNPV: *Bombyx mori* nucleopolyhedrovirus

B. mori: *Bombyx mori*

BrdU: 5-bromo-2-deoxyuridine

CDK: cyclin-dependent kinase

Col: colcemid

DCFH-DA: 2',7'-dichlorofluorescin diacetate

DDW: double distilled water

DMSO: dimethyl sulfoxide

Ecd: ecdysis

EGTA: o, o'-bis(2-aminoethyl)ethyleneglycol-N, N, N', N'-tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-related kinase

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

IVn: day n of the fourth larval stage

HCS: head capsule slippage

HU: hydroxyurea

IR: ionizing radiation

JH: juvenile hormone

JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase

LSC: laser scanning cytometer

MAPK: mitogen-activated protein kinase

PAGE: polyacrylamide gel electrophoresis

PI: propidium iodide

PMT: photomultiplier tube

PVDF: polyvinyliden difluoride

RNase: ribonuclease

ROS: reactive oxygen species

RT-PCR: Reverse transcription-polymerase chain reaction

SB: SB202190 (p38 inhibitor)

SD: standard deviation

SDS: sodium dodecyl sulfate

TRP: transient receptor potential

12L-12D: 12 h light and 12 h darkness

20E: 20-hydroxyecdysone

UV: ultraviolet

SUMMARY

Insect growth is greatly influenced by ambient temperature. Abnormal high temperatures induce growth arrest in insects but the causes are obscure. These stress temperature is not harmful to homoeothermic animals, indicating that the temperature sensitivity of insect growth is caused by special mechanisms for insects. However, it is difficult to examine the temperature effects on insect growth because of complexity of temperature reaction. To resolve this problem and study the temperature stress on insect cells, I used insect cell line that provides uniform experimental conditions and simplifies temperature reactions.

The influence of temperature on the BmN cell line which is derived from the silkworm, *Bombyx mori*, was investigated. These cells proliferated at an accelerated pace as the temperature increases from 22-30°C, but the growth rate slowed down at 34°C, and proliferation stopped at 38°C. At high temperatures, abnormal cellular morphology was observed. Cells treated at 38°C had cytoplasmic bi-lateral protrusions and they gradually aggregated and floated in the medium. BmN cells without proliferation at 38°C were viable but have reduced DNA synthesis. Laser scanning cytometry analysis revealed that at high temperatures, the cell cycle of BmN cells halted at the G_2 phase.

Next, the mechanisms of heat-induced G_2 arrest were examined. I focused the factors involved in transition from G_2 to M phase and analyzed the expression profile of cell cycle regulating genes by

real-time PCR. No significant difference was observed in their expression levels between 26 and 38°C, suggesting that the G_2 arrest is regulated in protein level. Because proceeding to M phase needs dephosphorylation of Cdc2 by Cdc25 phosphatase, the phosphorylation level of Cdc2 was examined by Western Blotting using phospho-Cdc2 specific antibody. The higher phosphorylation level which shows inactive state of Cdc2 was detected in the cells cultured at 38°C. The involvement of p38 MAPK activation by high temperature stress in the cell cycle arrest was confirmed using p38 specific inhibitor, SB202190.

The close relationship between high temperature stress and oxidative stress has been showed in many reports. In fact, 2',7'-dichlorofluorescin diacetate, which is an oxidation-sensitive fluorescent probe, showed the generation of reactive oxygen species (ROS) in BmN cells incubated at high temperature. From this result, I speculated that the oxidative stress occurred in heat-treated cells caused the cell cycle inhibition. This was verified by the recovery effect of antioxidant on the heat-induced cell cycle arrest. Treatment with ascorbic acid delayed p38 MAPK phosphorylation and suppressed G_2 arrest under high temperature. These results show that the generation of ROS by high temperature induces cell injury such as DNA damage and activates G_2/M checkpoint pathway in insect cells.

To confirm whether the inhibitory effects of high temperature observed in insect cell lines are occurred in silkworm cells, I investigated the influence of high temperature on the proliferation and

division of larval hemocytes. Although the total number of hemocytes in the larval body increased enormously over time at 26 °C, no increase of the cells was observed at 38 °C. The number of mitotic hemocytes in circulation increased between days 1 and 2 of the fourth larval stage at 26 °C, whereas fewer mitoses were observed at 38 °C. Laser scanning cytometry revealed that the DNA content of hemocytes collected from fourth-stadium larvae was predominantly 2C, 4C, and 8C, and the proportion of each type of hemocyte changed dynamically with development during the fourth instar. The proportion of hemocytes with a higher DNA content increased gradually during the feeding phase, then decreased during the molting phase at 26 °C, while no decrease of DNA content was observed at 38 °C. The heat-induced accumulation of 8C hemocytes was mainly detected in granulocytes and plasmatocytes. Furthermore, the level of phosphorylated Cdc2 increased in hemocytes from silkworms reared at high temperature. These data suggest that high temperature stress also induced G_2 arrest in larval hemocytes.

This study showed that abnormal high temperature for insects induced generation of ROS in insect cells and the oxidative damages cause G_2/M checkpoint pathway mediated by p38 activation. I conclude that the heat-induced cell cycle arrest is a major reason why insect growth is inhibited by high temperature stress.

GENERAL INTRODUCTION

Temperature has profound influence on living organisms and its change has marked effects on many physiological processes (Schmidt-Nielsen, 1997). Although temperature increase accelerates most process, living organisms have the range of temperatures they can tolerate. Exceeding the temperature range causes various damages to organisms and finally death. Insects are poikilothermic animal and therefore highly influenced by ambient temperature. Many researchers have been attracted to temperature reactions of insects and studied the reactions in various insect species (Chapman, 1998).

Influences of temperature are various and observed in whole insect life cycle and many phenomena. Diapause is a characteristic event of insect and it is interesting as a temperature reaction. To break diapause, diapausing insects must spend a certain period of time in a special condition such as low temperature, which is necessary for the termination of diapause (Andrewartha, 1952). Molting and metamorphosis are also attractive phenomena in insects and controlled by endocrine balance. Temperature affects on this endocrine balance. Wigglesworth (1972) found that low temperature upset the hormone balance slightly in favor of the juvenile hormone whereas high temperature slightly favors ecdysone. The emergence of species is timed so that the life history is synchronized with suitable environmental conditions and the meeting of the two sexes is facilitated.

Synchronization of biological event to the environment results from the common reaction of the members of a species to the environment. Temperature is particularly important in this point since to a large extent it governs the rate of development and the activity of the insect.

There is no good evidence that insects possess specialized temperature receptors. However, in grasshoppers the sensitivity to temperature is considered to be widely distributed over the body, although the antennae and tarsi are more sensitive than other parts (Chapman, 1998). Enzyme activity of insects increases with temperature. The greatest enzyme activity occurs at 45-50°C, but the activity is maintained only for short periods. Enzymes are denatured at high temperatures, and long periods over 40°C result in inactivation of this enzyme. Temperature reactions of insects are slightly different among the same species in their geographical distribution. Many strains of silkworm, *Bombyx mori* are maintained in Japan and other countries. Differences in the characteristic among the strains result from acclimatization to environment including temperature. All of the above-mentioned phenomena show that insects are closely related to temperature and indicate that insect life, especially the growth, is regulated by temperature. Temperature increase usually promotes insect growth but abnormal high temperature rather inhibits it.

Temperature effects on insects are complex and obscure. For example, when insects are reared at high temperature, the growth might be inhibited as a result of high temperature effects on the locomotion, feeding, digestion and so on. Endocrine and nervous system are also affected by high

temperature. It seems to be impossible to grasp all of these complex reactions caused by temperature. To resolve this problem, I focused on insect cultured cells. Many insect cell lines are established (Hink, 1970; Lynn, 1995; Imanishi *et al.*, 2003; Eguchi and Iwabuchi, 2006) and especially utilized in protein expression system (Murhammer, 1991; Altmann *et al.*, 1999). These cell lines maintain the properties of derived insect cells and are expected to have temperature reaction as well as the insect. Using insect cultured cells unifies the experimental conditions and simplifies the temperature reactions, and then the effects of temperature on insects would be understood in cellular level. Although many literatures about influences of temperature on insects exist, there is a little knowledge of temperature influences on insect cell lines.

In CHAPTER 1 of this thesis, I investigated the effects of temperature on BmN cell derived from silkworm, *Bombyx mori*. In Japan, *Bombyx* has been well studied because of flourish in sericultural industry and vast literatures have been accumulated. Furthermore, whole genome shotgun sequence was performed and silkworm genome database was enriched recently (Mita *et al.*, 2004). From these advantages, BmN cell was selected from many insect cell lines. BmN cells were usually cultured at 25-28°C, such as adequate temperatures to rear silkworms. I predicted that culture of BmN cells at various temperatures shows the direct effects of temperatures on insect cells. In fact, the arrest of cell cycle in G_2 phase was observed in stress temperatures (high and low temperatures).

In CHAPTER 2, I pursued the mechanism of cell cycle arrest under high temperature. Cell cycle

arrest in G_2 phase is often observed in cells exposed to stress conditions, such as ionizing radiation and osmotic shock (Dmitrieva *et al.*, 2001; de Vries *et al.*, 2005; Clotet *et al.*, 2006). The G_2 arrest is controlled by cell cycle checkpoint pathway (Pearce and Humphrey; 2001). Extreme high temperature (and extreme low temperature) is also considered to be one of stress conditions for insects. I therefore speculated that the checkpoint mechanism involved in heat-induced cell cycle arrest. I also examined whether the high temperature induced oxidative stress and the stress caused cell cycle arrest in insect cells, because it is argued that high temperature related to oxidative stress in many species (Harari *et al.*, 1989; Kim *et al.*, 2006).

Finally in CHAPTER 3, I verified the possibility that the heat-induced cell cycle arrest occurred in the silkworm. Hemocytes were used in this study since the cells are easy to isolate from silkworms and cell cycle analysis needs many separated cells. But there are two points to be considered in analysis in the silkworm. It is known that cell division in insects are regulated by molting hormone and fluctuates through the molting cycle (Gardiner and Strand, 2000; Koyama *et al.* 2004; Truman *et al.* 2006). In Addition, insects have many polyploid cells (Smith and Orr-Weaver, 1991; Edgar and Orr-Weaver, 2001). These are serious problems for cell cycle analysis. To understand proliferation pattern and DNA content of silkworm hemocyte is important for examination of high temperature effects on hemocyte cell cycle. I first cleared the proliferation pattern of hemocyte in the fourth larval stage and then the effects of high temperature on the

hemocytes were assessed. From all results, I conclude that stressful high temperature for insects induces oxidative stress in the cells and activate G_2/M checkpoint pathway.

CHAPTER 1

High Temperature Causes Arrest of Cell Cycle in G_2 Phase in BmN Cells Derived from the Silkworm, *Bombyx mori*

INTRODUCTION

The close relationship between insects and temperature has been studied extensively, with, most studies observing of insect behavior at low and high temperatures, or insect resistance to heat and cold (Chapman, 1998). The occurrence of diapause and physiological adaptations in insects exposed to low temperatures have also been examined in detail (Cymborowski, 2000; Denlinger, 2002). Heat shock protein is discovered in *Drosophila melanogaster* while investigating tolerance mechanisms against high temperature (Ashburner and Bonner, 1979), and the heat shock responses of different thermotolerant races of the mulberry silkworm, *Bombyx mori* have been also reported (Joy and Gopinathan, 1995). The developmental period of insects is known to shorten in response to increased temperatures, and this relationship has been examined in a number of insect species, and equations relating environmental temperature to developmental time have been constructed (Wigglesworth, 1972). The relationship between the rearing temperature and growth of *Bombyx mori* has been investigated by Takamiya and Nakajima (1970). Sudo *et al.* (1999) formulated equations

representing the relationship between rearing temperature and growth of silkworm larvae, and calculated the low temperature limit for growth. These studies show that insect growth is regulated strictly by temperature but the molecular mechanism of regulation has not been elucidated.

Attaining control of experimental conditions is difficult when investigating the effects of temperature on insects because factors other than temperature also affect insect growth. In particular, humidity and food deterioration are critical factors. Accordingly, to investigate the relationship between temperature and insect growth, cultured cells were used instead of whole insects to provide a stable experimental condition. The use of cultured cells can simplify the experimental system and understanding of insect cell reactions to temperature at the cellular level and can help to elucidate the relationship between temperature and insect growth at the molecular level.

BmN cells, an established cell line derived from *Bombyx mori*, were cultured at various temperatures, and the cell proliferation, viability, and DNA synthesis were compared. Cell cycle analysis using a laser scanning cytometer (LSC) showed G_2 arrest of the cells at high temperature.

MATERIALS AND METHODS

Silkworm and cell line

The silkworm, *Bombyx mori* Shoon, was used in this study. Larvae were reared on an artificial diet (Nihonnosankogyo Co., Japan) in a LD 12: 12 h photocycle at 25 ± 2°C. Groups of larvae at day 0 of the fourth larval stage (10 males and 10 females) were reared in plastic cases (20.5 × 15.0 × 5.2 cm) at various temperatures between 10-38°C without light. Fluctuations of the temperature and humidity in the incubators were controlled within 0.5°C and 60-70%, respectively. The diets were exchanged every day to avoid deterioration, and individual larval weights were measured until ecdysis to the fifth instar. For observation of hatching, the eggs treated with artificial hatching were incubated for 30 days at 10-38°C without light. For observation of adult emergence, newly ecdysed pupae were transferred to the incubators set at 10-38°C and maintained for 30 days without light.

The BmN (BmN4) cell line derived from *Bombyx mori* (Maeda, 1989) was maintained in TC-100 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% antibiotic antimycotic (Sigma-Aldrich Co. Ltd., Irvine, UK) at 25°C.

Analysis of cell growth

Cell density was estimated by counting the cells from a sample of the suspension in hemocytometer under a microscope. The culture was initiated by seeding a 96-well plastic plate with

1×10^4 cells /well, and the plate was sealed to prevent desiccation. The cells were maintained at 25°C for 2 days to promote cellular attachment to the well. After this preculture, the cells were moved into incubators set at 10-38°C. Temperature fluctuations were controlled to within 0.5°C. Cultured cells were fixed with 5% formaldehyde every 24 h for 5 days. The cells were centrifuged at 200 × g for 10 min to attach the cells to the bottom of the well, and the medium was removed. For staining, 50 µl of 0.1% crystal violet (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution were added to each well and incubated for 10 min at room temperature. Stained cells were washed twice with distilled water and vortexed in 100 µl of 10% SDS solution. The absorbance of each well at 550 nm was measured using a Microplate Imaging System (Bio-Rad, Laboratories, CA, USA). Absorbance increased linearly in proportion to cell density (regression coefficient = 0.997). Cell density was estimated by comparison with a standard curve made by serial dilution of BmN cells counted with a hemocytometer.

Cellular morphology and viability

The cellular morphology was observed in an inverted light microscope and was photographed using a microscope digital camera (DP12; Olympus, Tokyo, Japan). Cell viability was determined using the trypan blue dye exclusion method (Altman *et al.*, 1993). A trypan blue solution (0.4% wt/vol, Gibco Co., Grand Island, NY, USA) was mixed with an equal volume of cell suspension and

maintained at room temperature for 3 min. The suspension was loaded on a hemocytometer, and stained cells were scored as nonviable.

DNA synthesis assay

De novo DNA synthesis was measured by the incorporation of 5-bromo-2-deoxyuridine (BrdU) using the Cell Proliferation Biotrak ELISA System, version 2 (Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK). The final concentration of BrdU in each well was 10 μM.

Cell cycle analysis

BmN cells cultured at various temperatures between 10-38°C in 6-well plates were suspended gently, and 100 μl of the suspension was placed on slides coated with poly-Lysine (POLY-PREP™ SLIDES; Sigma-Aldrich). Cells were fixed with Carnoy fixative (ethanol: chloroform: acetic acid, 2:1:1) for 10 min, and the fixative was removed by pipette. The cells were treated again with 100 μl of Carnoy fixative and dried. The fixed cells were washed twice using 100 μl of PBS (phosphate-buffered saline; 10 mM Na_2HPO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.4), 100 μl of the solution containing 50 μg/ml RNase (Ribonuclease; Roche Diagnotics Co., Indianapolis, IN, USA) and incubated with 5 μg /ml PI (propidium iodide; Sigma) for 1 h at 37°C. These cells were mounted with a cover glass using Vectashield (Vector Laboratories, Burlingame, CA, USA) and the

fluorescence was measured using a laser scanning cytometer (LSC) (Olympus; Darzynkiewicz *et al.*, 1999; Luther and Kamentsky, 1996). PI fluorescence (610-620 nm) was excited using an argon laser at 488 nm. An average of 5000 cells on a slide was scanned automatically using a 20× objective lens. At the initiation of scanning for each slide, the sensitivity of the threshold photomultiplier tube (PMT) was adjusted so that maximum detection of the stained areas was achieved with minimum detection of background staining.

RESULTS

The relationship between temperature and the growth rate of silkworm

Body weights were recorded of fourth-instar larvae of the silkworm reared at 10-38°C until they ecdysed to the fifth instar (Fig. 1-1). The growth rate during the feeding phase increased with temperature between 10 and 26°C and then reached a plateau. The growth rate unchanged largely at 26-34°C but at 38°C the weight gain was lower than at these temperatures. When the weight of silkworm reached about 0.5 g, the larvae stopped feeding and entered the molting phase. Ecdysis to the fifth-instar larvae occurred normally at temperatures between 22 and 30°C, but in silkworms reared at 34°C, they failed to enter the molting phase and grew beyond the limits of the weight in normal fourth-instar larvae. After rearing at 38°C for 4 days, silkworms weakened gradually and died without entering the molting phase. Molting was not observed for 5days at temperature between 10 and 18°C, but long term incubation at 18°C induced larval molting.

Effects of temperature on the silkworm development were examined (Fig. 1-2). Artificial hatching-treated eggs hatched 11 days after the treatment at 26°C. The embryo developmental rate was accelerated by temperature increase but no larvae hatched for 30 days at below 14°C and above 34°C. Newly ecdysed pupae were incubated at the various temperatures and the adult emergence was observed. Although the days needed for emergence were shortened by temperature increase, no pupae emerged for 30 days at above 34°C. No emergence was also observed at below 14°C. Pupal

development to adults was observed at 34°C but not at 38°C.

Effect of high temperature on BmN cell growth and morphology

The BmN cell line was cultured at various temperatures, and the cell numbers were counted. In temperatures ranging from 10-26°C, the growth rate increased constantly as temperature increased (Fig. 1-3). Growth rates calculated from cell numbers between day 0 and day 3 were 0.26, 0.36, 0.73, 1.27, 1.89, 1.88× 10^5 cells/ml/day at 10, 14, 18, 22, 26, and 30°C, respectively. At 26 and 30°C, cell density reached a maximum level of about 1.5 × 10^6 cells/ml after incubation for 5 days. Cells that were cultured at 34°C proliferated initially at day 2, but the growth rate declined at day 3, and the proliferation was suppressed thereafter. Cell proliferation was inhibited entirely by incubation at 38°C. The number of the cells did not increase at 42°C (date not shown).

BmN cells cultured at 18-30°C were spherical and showed normal morphology (Fig. 1-4A). After incubation for 12 h at 38°C the cells elongated like fibroblasts in appearance, with cytoplasmic protrusions positioned in a bi-lateral (Fig. 1-4B). Elongated cells returned to a normal state and began to proliferate when the temperature was lowered to 26°C. The number of elongated cells increased steadily at 34°C. Cells incubated at 34 or 38°C for 4 days aggregated gradually and contained many granules (Fig. 1-4C). The cells detached from the bottom of the wells and floated in the medium after such prolonged incubation at these high temperatures. Although the shapes of cells

remained unchanged, the cell membrane denatured after incubation for 12 h at 42°C (Fig. 1-4D) and did not revert to normal when cells were moved to 26°C. Long term culture at 10 or 14°C caused the cells elongation similar to that observed at high temperature (data not shown).

BmN cell viability and DNA synthesis at high temperature

Cell viability was examined using the trypan blue exclusion method (Fig. 1-5). All cells incubated at 42°C showed an abnormal cell membrane, but only 20% of the cells were stained at day 1. The number of nonviable cells increased with incubation time, and almost all cells were stained after 4 days. Although the cells showed morphological abnormalities, the number of stained cells remained under 10% after 4 days at 34 and 38°C, but reached 20% at day 5. Between 22 and 30°C, the number of stained cells remained below 5%. The number of nonviable cells exceeded 90% in 1 day when cells were incubated at 46°C (data not shown).

The amount of BrdU incorporated into newly synthesized DNA at various temperatures was examined to gauge DNA synthesis in the process of cell division (Fig. 1-6). BmN cells were cultured at 25°C for 2 days on a 96-well plate, and the BrdU solution was added to each well. After the cells were incubated at different temperatures (22-38°C) for 24 h, the incorporated BrdU was measured immunochemically. No significant difference was observed between 22 and 34°C, but BrdU incorporation at 38°C was reduced to approximately 30% of levels at other temperatures (Fig. 1-6A).

When BmN cells were cultured at 30-38°C for 100 h before the addition of BrdU, a 70% decrease was observed at 34°C, and the cells did not incorporate BrdU at 38°C (Fig. 1-6B).

Cell cycle G_2 phase arrest at high temperature

The results described above suggested that cellular proliferation ceased at high temperatures in response to an arrest of the cell cycle and not because of cell death. Therefore, I analyzed the cell cycle with an LSC to define the point of arrest of cell division (Fig. 1-7). When plotted as scattergrams with the abscissa representing the integrated fluorescence, which indicates DNA content, and the ordinate representing maximum pixel fluorescence, the height representing the number of cells, the fraction of the cell population in G_1, S, G_2, and M phases of the cell cycle could be identified visually. The largest percentage of the cell population, *c.* 50%, was observed in the G_1 phase, and 30% was in the G_2 phase at 26°C. These proportions were maintained over the entire culture period at 26°C. In contrast, cells in the G_2 phase accumulated after 24 h of incubation at 38°C (G_1: 25%, G_2: 50%), and the population of G_2 cells increased when incubation was prolonged. The fraction of G_2 cells returned to the usual distribution when the temperature was lowered from 38 to 26°C. Regardless of the temperature, the S phase and M phase were almost constant at about 20% and 5%, respectively.

Cell cycle at various temperatures

Furthermore, the cell cycle analysis was performed in BmN cells incubated for 72 h at various temperatures. As shown in Figure 1-8, the percentages of the cell population in G_1, S, and G_2/M phases of the cell cycle were maintained after cultivation for 72 h at 26°C (G_1: 51%, S: 17%, G_2/M: 32%). The percentage of cells in G_2 increased gradually toward both low and high temperature. A high proportion of the cells cultured at 38°C remained in G_2 phase (about 70%), while only a small proportion of the cells remained in G_1 (about 9%). Cells in G_2 phase were observed in 54% of incubated cells at 10°C, resulting that G_2 block of the cell cycle was also caused by low temperature stress.

DISCUSSION

Similarity of temperature reaction between silkworm and the cell line

Established insect cell lines are generally thought to retain some properties of the original insects. The concept probably applies to the reaction of cells to temperature because the temperatures of expression for heat shock proteins differ in various insect cell lines (Gerbal *et al.*, 2000). The present results suggest that the response of BmN cells to temperature is related closely to that of silkworm larvae. In both the larval weight gain and proliferation of BmN cells, increases of growth rate with temperature between 10 and 26°C were observed (Figs. 1-1 and 1-3). This result shows that the optimal temperature for silkworm and the cell line growth is approximately 26°C. Disturbance of the hatching, molting, and emergence occurred in *Bombyx* reared at below 14°C and above 34°C (Figs. 1-1 and 1-2). Cell cycle arrest was also induced by cultivation at these low or high temperatures (Fig. 1-8).

Heat-induced cell cycle arrest in insect cell line

The growth of BmN cells stopped at 38°C (Fig. 1-3), and the BrdU incorporation assay indicated that DNA replication was reduced in these cells (Fig. 1-6). The cell viability test with trypan blue, however, showed that the non-proliferating cells did not completely lose their viability (Fig. 1-5). Moreover, with cells at 38°C for short periods, the morphology returned to a normal state and

proliferation re-started after they were transferred to 26°C. These results suggest that the cells are hindered by high temperature and cell division stops at a particular cell cycle phase before death. It was found that the cells arrest in the G_2 phase of the cell cycle (Fig. 1-7). LSC analysis showed that the highest fraction of cells was at the G_1 phase after incubation at 26°C, but G_2 cells were abundant at 38°C. When the temperature was lowered to 26°C, the number of cells at the G_1 phase increased again.

Therefore, the arrest of the cell cycle at the G_2 phase in response to inadequate conditions is a possible mechanism used by insect cells to stop cellular development. Fertig *et al.* (1990) reported that the resting phase of insect cells was characterized by 4c-DNA content, whereas the G_0 phase of mammalian cells was characterized by 2c-DNA content. Doverskog *et al.* (2000) showed that synchronization of the cell population in the G_2/M phase of the cell cycle occurred initially during the lag phase and toward the end of the population growth phase in a serum-free batch culture of *Spodoptera frugiperda* (Sf9) cells. Arrest of BmN cells at G_2/M phase happens in *Bombyx mori* nucleopolyhedrovirus infection (Baluchamy and Gopinathan, 2005). The accumulation of BmN cells in the G_2 phase was also observed in a confluent state (unpublished data). Furthermore, low temperature stress also caused an increase in the population of cells in G_2 phase (Fig.1-8). Although each of the signal pathways that induce cell cycle arrest is variable, the downstream mechanisms, such as G_2 arrest, may be identical in insect cells.

The application of 20-hydroxyecdysone induces a sharp decrease in the level of cyclin A and B expression and arrest the cell cycle of lepidopteran IAL-PID2 cells in the G_2 phase (Mottier *et al.*, 2004). It can be speculated that the expression of cell cycle regulation genes fluctuates also during the G_2 arrest at high temperatures. The analysis of gene expression is expected to reveal the mechanism of cell cycle arrest caused by high temperatures.

High temperature could induce excessive consumption of nourishment or accumulation of waste products in the culture and stop the cell cycle indirectly. However, the recovery from cell cycle arrest in the absence of medium exchange refutes this possibility.

Heat-induced damages to insect cultured cells

At least two reactions to high temperature occur in cultured cells: one is the arrest of cell proliferation, and the other is cell death. The differences in cell morphology that occurs between 38 and 42°C indicate the occurrence of these two reactions (Fig. 1-4). When the temperature was reduced to 26°C, cells that had been elongated at 38°C reverted to a native morphology, while cells with abnormal cell membranes at 42°C could not revert to a normal morphology. Component proteins of the cell membrane are probably destroyed at 42°C. The percentage of nonviable cells increased rapidly with time at 42°C, and almost all cells died within 1 day at 46°C. Although damages to the proliferation, morphology, and cell cycle were not observed initially in BmN cells

cultured at 34°C, longer exposure gave the cells serious damages. The principle that the more the temperature increases, the more rapidly the effects of unfavorable temperatures for organisms arise (Schmidt-Nielsen, 1997), is applicable to the cultured insect cells. When BmN cells that had ceased proliferating were transferred to 26°C and incubated again, cells that had been incubated for shorter times at 38°C returned to the normal cell cycle (Fig. 1-7). An extended incubation time at 38°C induced serious morphological changes and reduced cell number followed by an increment in the numbers of nonviable cells. Perhaps the cells were gradually degraded by necrosis or apoptosis, because much cell debris was observed in the medium after long term incubation at 38°C. The reduction in cell viability at 38°C may be caused by long-term arrest of the cell cycle.

FIGURES AND TABLES

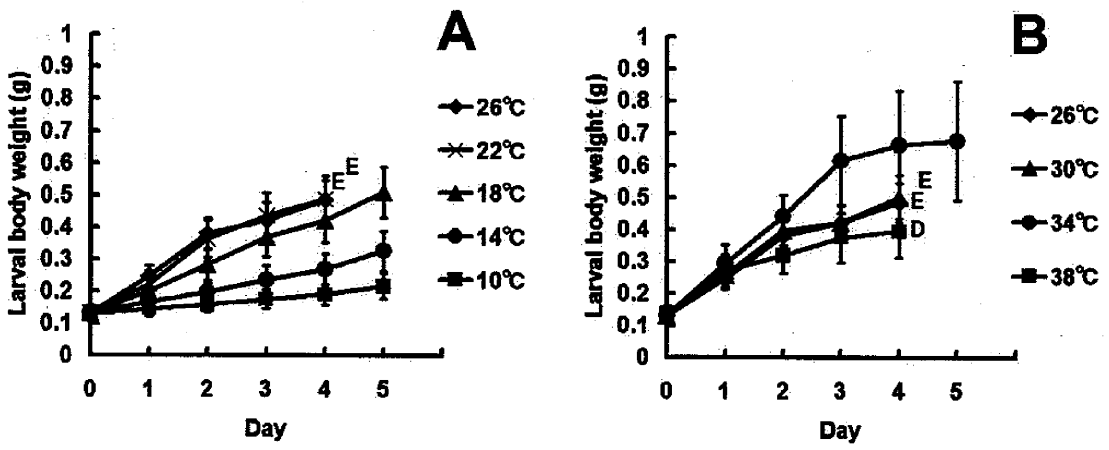

Figure 1-1

The effects of rearing temperature on the weight gain in fourth-instar larvae of the silkworm. (A) The range of low temperatures. (B) The range of high temperatures. Fourth-instar larvae were weighed on an electronic balance to the nearest 1 mg. Data points are averages ± SD, n = 20. E: Ecdysis, D: Dead.

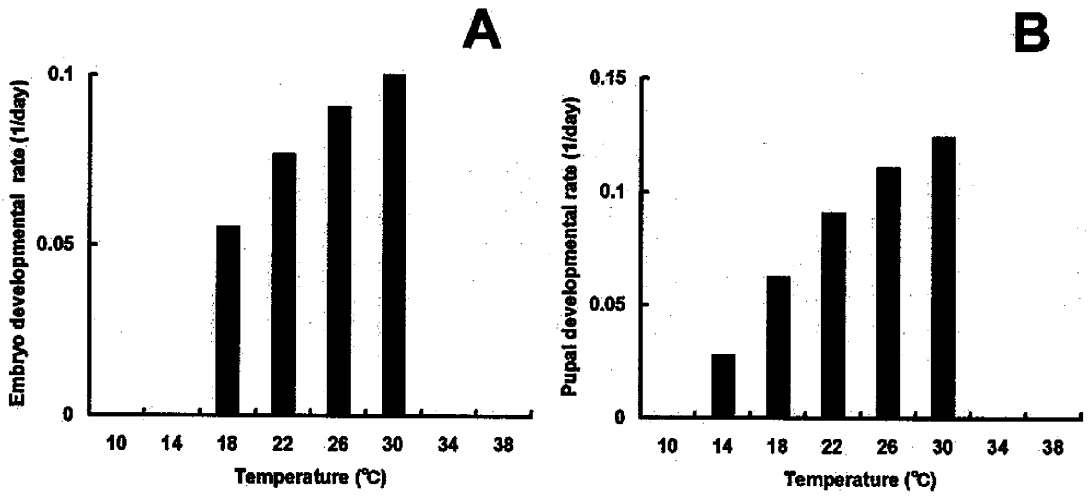

Figure 1-2

The effects of temperature on the silkworm development. (A) Diapause eggs treated with artificial hatching were incubated at indicated temperatures for 30 days without light. Hatching day was determined when more than 50% of larvae hatched ($n > 20$). Embryo developmental rate was represented by the reciprocal number of hatching day. (B) Newly ecdysed pupae were incubated at indicated temperatures for 30 days without light. Emergence day was determined when more than 50% of pupae emerged ($n = 10$). Pupal developmental rate was represented by the reciprocal number of emergence day.

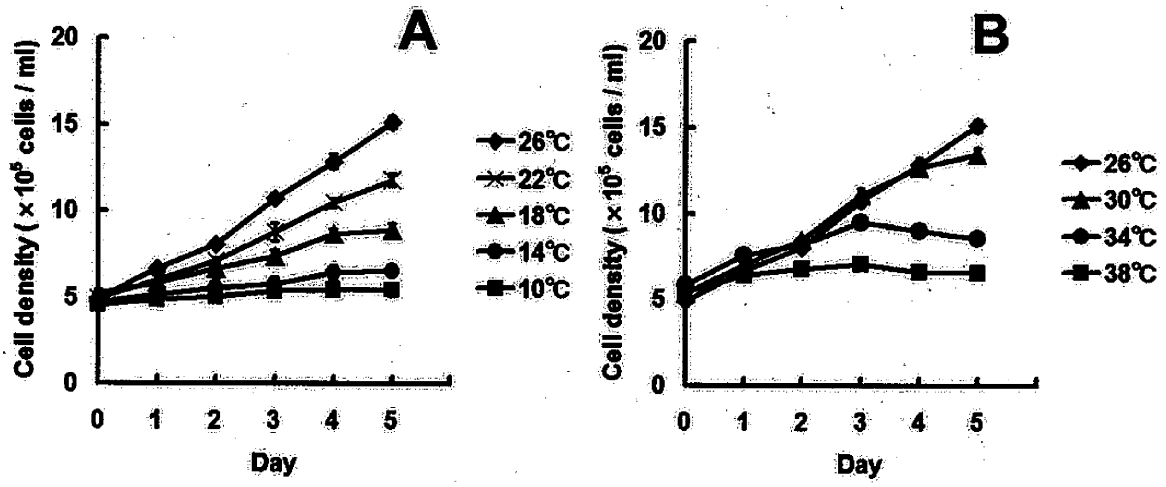

Figure 1-3

Proliferation of BmN cells at various temperatures. (A) The range of low temperatures. (B) The range of high temperatures. Cell density was determined by crystal violet-SDS methods. Data points are averages ± SD, n = 6.

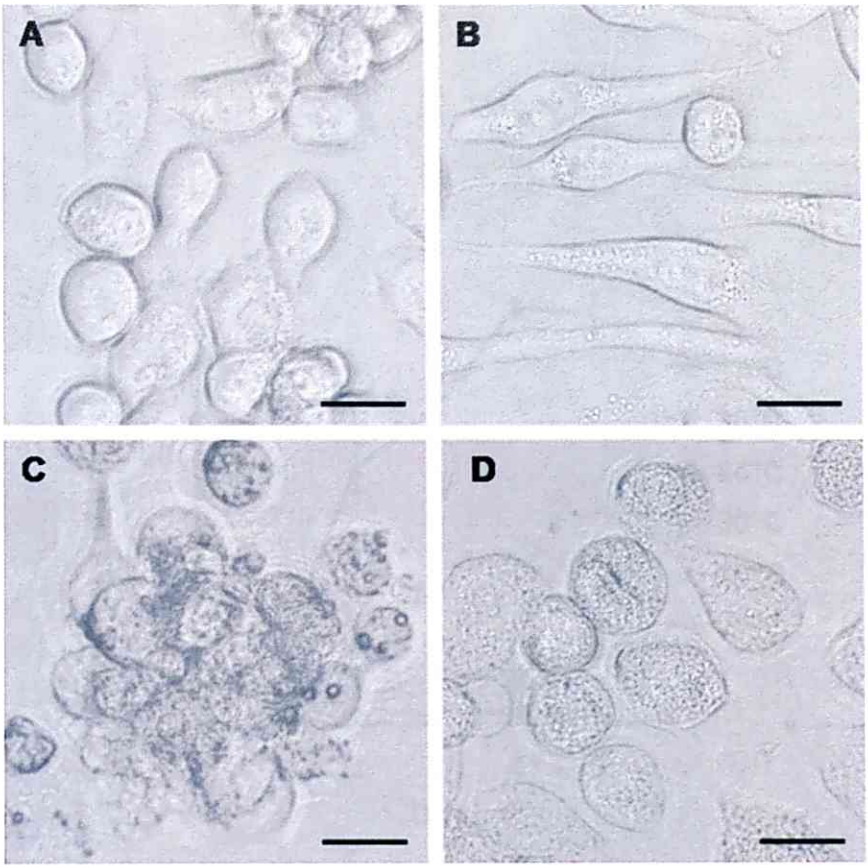

Figure 1-4

BmN cell morphology at normal and high temperatures. (A) 26°C, (B) 12 h at 38°C, (C) 96 h at 38°C and (D) 12 h at 42°C. Scale bar = 20 μm.

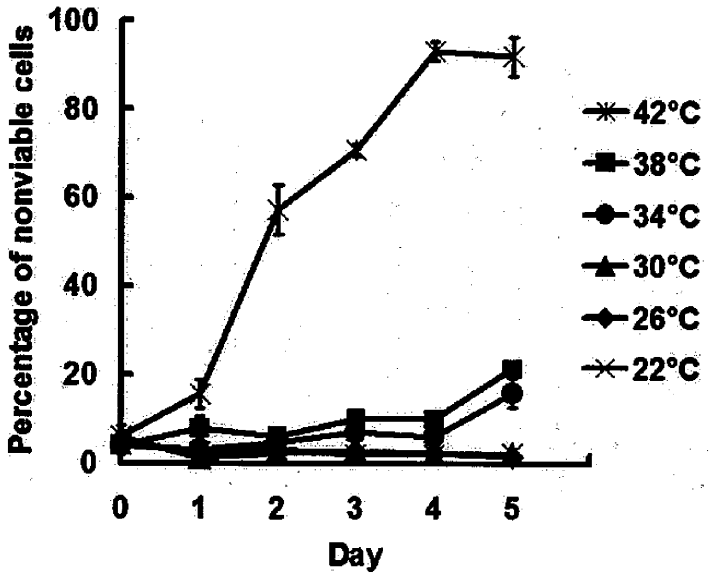

Figure 1-5

Effect of temperature on BmN cell viability. The percentage of nonviable cells was determined using the trypan blue dye exclusion test. Data points are averages ± SD, n = 3.

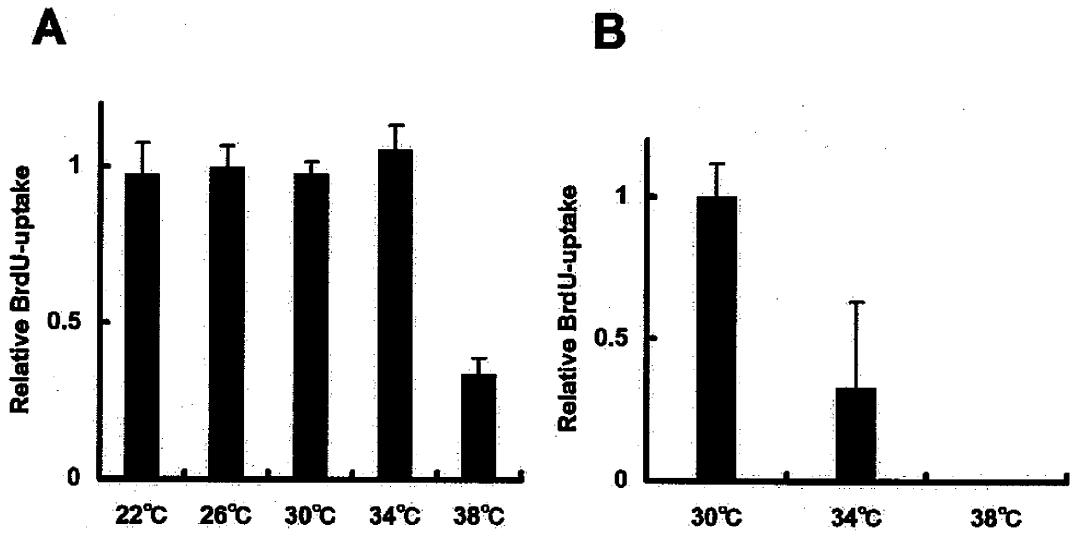

Figure 1-6

Arrest of DNA synthesis in BmN cells at high temperature. (A) BrdU was added to the culture medium, and the cells were incubated for 24 h at various temperatures. (B) Cells were cultured for 100 h at indicated temperatures without BrdU, and then for 24 h with BrdU added. Each value is the average ± SD of six different cultured wells; the values are corrected by cell counts. The relative level of BrdU-uptake compared with that at the normal temperature (26 or 30°C) was defined as 1.

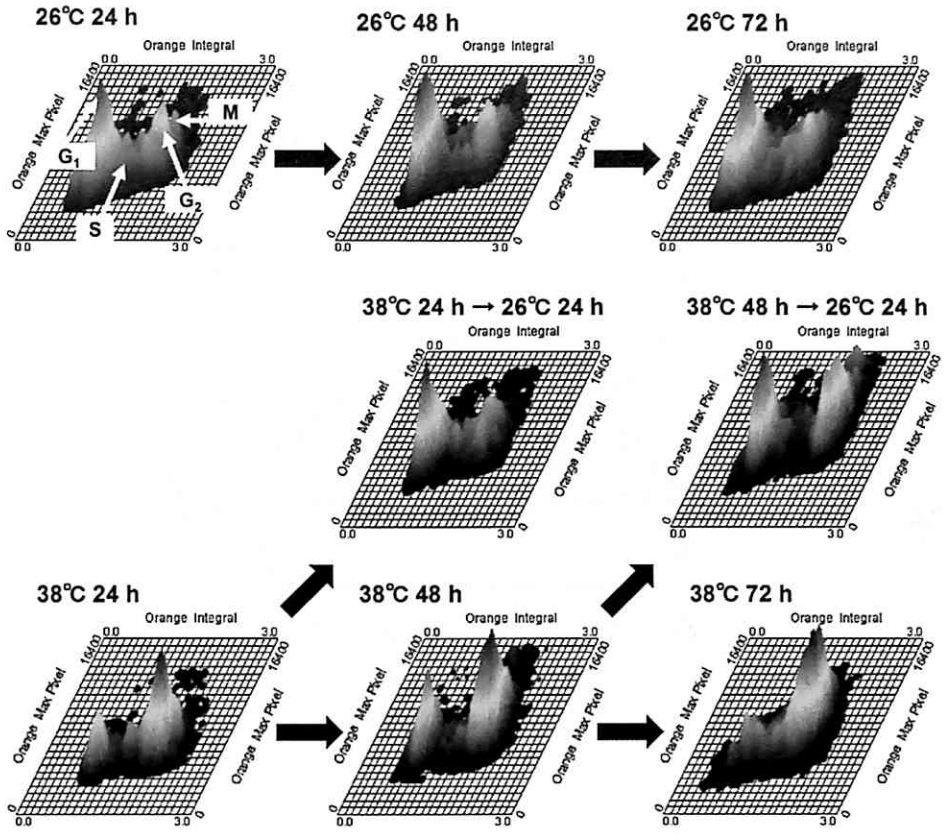

Figure 1-7

Cell cycle analysis of BmN cells after incubation at 26 and 38°C. The 3D histograms were drawn using LSC software. Abscissa: DNA content, ordinate: degree of chromatin condensation, height: number of cells. White arrows indicate phases of the cell cycle.

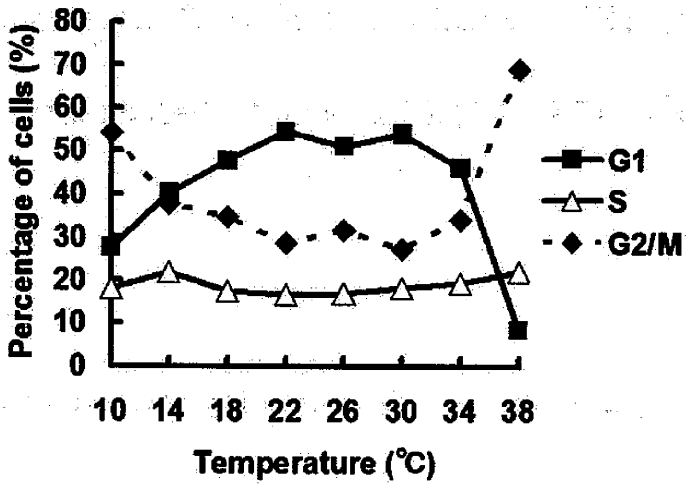

Figure 1-8

Distribution of cell cycle at various temperatures. BmN cells were incubated for 72 h at indicated temperatures and then laser scanning cytometry analysis was performed.

CHAPTER 2

High Temperature Induces Accumulation of ROS and G_2 Cell Cycle Arrest through Activation of p38 MAPK in Insect Cell Line

INTRODUCTION

The insect is a representative poikilothermic animal and reacts acutely to ambient temperature. Although insect growth is accelerated by temperature increase, the growth is inhibited by abnormal high temperatures for insects. These abnormal temperatures are rather adequate to homoeothermic animals such as mammals and birds. This difference of favorable temperature shows that insects are more sensitive to temperature stress than mammals but the reasons are obscure. In general, it is speculated that harmful effects of high temperature are probably caused by enzymatic inactivity, behavior disorder, and water loss, etc. The detailed mechanisms, however, are unknown.

Many insect cell lines are established (for examples, see Hink, 1970; Lynn, 1995; Imanishi *et al.*, 2003; Eguchi and Iwabuchi, 2006). The growth rates of BmN cell derived from the ovary of silkworm, *Bombyx mori*, were affected by temperature as well as that of silkworms (CHAPTER 1). Our challenge using insect cell lines to elucidate the effects of temperature on insects in cellular level suggested that insect cells had G_2/M checkpoint mechanism of cell cycle against high

temperature stress. However, mediators of this signal cascades are still unclear.

The p38 stress kinase respond to various environmental stimuli, including ultraviolet (UV) radiation, oxidative stress, protein synthesis inhibitors, heat shock, cytokines and osmotic shock (Ono and Han, 2000; Kyriakis and Avruch, 2001). Following their activation through phosphorylation of highly conserved neighboring tyrosine and threonine residues, p38 kinases can localize to the nucleus, where they phosphorylate and activate their target transcription factors (Pearce and Humphrey, 2001). In fission yeast, osmotic stress, treatment with hydrogen peroxide (H_2O_2), heat shock, and UV radiation activate the Spc1/Sty1 stress kinase pathway (Millar, 1999). Spc1/Sty1 is a homolog of human p38 and budding yeast Hog1 MAPK (mitogen-activated protein kinase). In fruit fly, p38 have been cloned, and its tyrosine 186 is rapidly phosphorylated in response to osmotic stress, heat shock, serum starvation, and H_2O_2 in *Drosophila* l(2)mbn and Schneider cell lines (Han *et al.*, 1998). Furthermore, flies lacking p38 are susceptible to some environment stresses including heat shock, oxidative stress and starvation (Craig *et al.*, 2004). On the other hand, roles for stress kinases in cell cycle control are reported. Exposure of yeast to osmotic stress leads to activation of the Hog1 SAPK (stress-activated protein kinase) and G_2 arrest (Clotet *et al.*, 2006). Vanadium induces generation of H_2O_2 and superoxide radical, and then stimulates MAPK family members to arrest cell cycle at the G_2/M phase in the human lung alveolar epithelial cancer cell line A549 (Zhang *et al.*, 2003). Bulavin *et al.* (2001) reported that p38 kinase had critical role in the

initiation of a G_2 delay after UV radiation. These reports suggest that activation of p38 MAPK by environmental stress including heat shock has close relationship to G_2/M checkpoint pathway.

Heat stress induces generation of reactive oxygen species (ROS) such as the H_2O_2, superoxide anion and hydroxyl radical (Harari *et al.*, 1989; Kim *et al.*, 2006) and the ROS damage to protein, lipid membranes, and DNA. Oxidative stress increases heat shock proteins, while over-expression of antioxidant enzymes caused an increase in thermotolerance (Davidson *et al.*, 1996). These reports suggest that there are close relationship between heat stress and oxidative stress. In the case of insects including *Bombyx*, exposure to high temperature activates some antioxidant enzymes in transcriptional and translational level (Lee *et al.*, 2005; Kim *et al.*, 2007).

In the present study, I propose that heat stress induces G_2/M checkpoint in insect cells through activation of p38 and following inactivation of Cdc2. In addition, I elucidate that the activation of p38 is resulted from unexpected accumulation of ROS in insect cells at high temperature.

MATERIALS AND METHODS

Cell line

The BmN (BmN4) cell line derived from *Bombyx mori* (Maeda, 1989) was maintained in IPL-41 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1% antibiotic-antimycotic (Sigma-Aldrich Co., Ltd., Irvine, UK) at 25°C. The cell number was counted in a defined area (eight random areas, 3.03×10^{-3} cm^2 each) under an inverted microscope (IX70, Olympus, Tokyo, Japan) and the total number of the cells in a well was calculated.

Reverse transcription (RT) and real-time quantitative polymerase chain reaction (PCR)

Total RNA from BmN cells was isolated using ISOGEN (Wako Pure Chemical Industries). The RNA was measured to confirm its purity and stability with a GeneQuant pro (Amersham Pharmacia Biotech, Uppsala, Sweden). Five μg of total RNA in 20 μl volume were converted to cDNA by use of ReverScript IV (Wako Pure Chemical Industries) and an Oligo(dT) 12-18 primer (Invitrogen), according to the manufacture's protocol. After incubation at 37°C for 30 min with 1μl of Ribonuclease H (TaKaRa Biotechnology Co., Ltd., Shiga, Japan), the cDNA was purified by ethanol precipitation and dissolved in 200 μl of double distilled water (DDW). One μl of RT product was amplified by real-time PCR using the SYBR Premix ExTaq Perfect Real-Time PCR Kit (TaKaRa),

according to the manufacture's protocol. The primers used are shown in Table 2-1.

Western blotting

Cells were rinsed with cold phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.4), then lysed with SDS-sample buffer (50 mM Tris-HCl: pH 6.8, 2% sodium dodecyl sulfate, 0.86 M 2-mercaptoethanol, 10% glycerol). After the samples denatured by boiling, the protein amount was measured by using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was conducted by the method of Laemmli (Laemmli, 1970). Twenty five μg of proteins were analyzed in 10% polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA, USA) using transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The membranes were blocked for 1 h at room temperature in TBS/T buffer (0.1% Tween-20, 25 mM Tris, and 150 mM NaCl, pH 7.6) containing 2% ECL Advance blocking agent. The blotted membranes were washed three times for 5 min each with TBS/T buffer and then incubated overnight at 4°C with each primary antibody. Used antibodies were rabbit anti-phospho-Cdc2 (Sigma, St Louis, MO, USA), anti-Cdc2 (PSTAIRE, Upstate Biotechnology, Lake Placid, NY, USA), anti-phospho-p38 (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-phospho-ERK (Cell Signaling Technology), anti-phospho-JNK/SAPK (Cell Signaling Technology) and mouse anti-α-tubulin (Sigma). They were diluted at 1:2000 in TBS/T

containing 5% bovine serum albumin. After incubation with the primary antibodies, the membranes were washed five times with TBS/T. Secondary antibodies, horse radish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology) diluted at 1:4000 in TBS/T buffer containing 2% ECL Advance blocking agent, were then added and further incubated for 1 h at room temperature. The membranes were then washed five times in TBS/T. Immunoreactivity was visualized using an ECL Advance Western Blotting Detection Kit (Amersham Biotech, Little Chalfont Buckinghamshire, UK) and LAS-1000 Luminescence Image Analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). Densitometric analyses were conducted through use of a PC software, Scion Images for Windows (Scion Co., Frederick, MD, USA).

Cell cycle analysis

BmN cells cultured on cover slip in 6-well plates were rinsed gently with PBS, and fixed with Carnoy fixative (ethanol: chloroform: acetic acid, 2:1:1) for 10 min. After the cells were washed three times using PBS, 100 μl of the solution containing 50 μg/ml RNase (Ribonuclease; Roche Diagnotics Co., Indianapolis, IN, USA) and 5 μg/ml PI (propidium iodide; Sigma) was placed on the cells and incubated for 1 h at 37°C. The cells were washed three times, and then mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). The fluorescence was measured using a laser scanning cytometer (LSC; Olympus; Luther and Kamentsky, 1996; Darzynkiewicz *et al.*, 1999).

PI fluorescence (610-620 nm) was excited using an argon laser at 488 nm. An average of 5000 cells on a slide was scanned automatically using a 20× objective lens. At the initiation of scanning for each slide, the sensitivity of the threshold photomultiplier tube (PMT) was adjusted so that maximum detection of the stained areas was achieved with minimum detection of background staining.

Detection of intracellular reactive oxygen species (ROS)

Fluorescence microscope (BX51; Olympus) and CCD camera (DP50; Olympus) were used to measure the generation of ROS by detecting 2',7'-dichlorofluorescin diacetate (DCFH-DA; Sigma), an oxidation-sensitive fluorescent probe. The principle of this assay is that DCFH-DA diffuses into the cell through the membrane and is enzymatically hydrolyzed by intracellular esterase to nonfluorescent dichlorofluorescin. In the presence of ROS (mainly H_2O_2), this compound is rapidly oxidized to highly fluorescent dichlorofluorescein (Bass *et al.*, 1983; LeBel *et al.*, 1992; Zhang *et al.*, 2003). BmN cells were cultured in 35 mm dish and received temperature treatments. The incubated medium was exchanged to new one preincubated at 26°C, and then DCFH-DA was added to the cells to a final concentration at 50 μM. After incubation for 30 min at 26°C, the cells were washed with insect PBS (4.5 mM Na_2HPO_4, 137 mM NaCl, pH 6.5) three times and immediately analyzed with a fluorescence microscope. To examine the direct effects of H_2O_2, the cells were preincubated

with 50 μM DCFH-DA for 1 h at 26°C and then treated with 100 μM H_2O_2 for 30 min. After the incubation, the cells were washed and analyzed with a fluorescence microscope.

Chemicals

SB202190 (p38 inhibitor) was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). L(+)-ascorbic acid (vitamin C; Wako Pure Chemical Industries) and hydroxyurea (Sigma) were dissolved in DDW. Colcemid (demecolcine) was purchased from Wako Pure Chemical Industries and dissolved in DMSO.

Statistics analysis

The results are presented as representative experiments or as means ± SD (n = 3). The statistical significance of differences was determined by one-way ANOVA, followed by Scheffe's *F*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Expression profiling of cell cycle genes

I attempted initially to check the expression level of cell cycle genes related particularly to G_2/M phase by real-time PCR, because a silkworm cell line, BmN cell, was arrested at G_2 phase of cell cycle by high temperature stress (CHAPTER 1). There was no significant difference between 26°C (normal temperature for insects) and 38°C (stress condition) in the expression level of cell cycle genes participating in G_2/M phase (cdc2, cyclin A, cyclin B, and cdc25; Fig. 2-1). The expression level of G_1 cyclin (cyclin D) and housekeeping gene (ribosomal protein 49; rp49) also unchanged extremely under high temperature. Heat shock protein 70 (hsp70) was four times increased by heat treatment.

The effect of high temperature on Cdc2 phosphorylation

Cdc2 is a key regulator of cell cycle. To proceed from G_2 to M phase, activation of Cdc2 by dephosphorylation on Thr14/Tyr15 is needed (Morgan, 1995). In this reason, the phosphorylation level of Cdc2 under high temperature stress was examined by Western blotting. Specificity of anti phospho-Tyr15-Cdc2 antibody to *Bombyx* Cdc2 was confirmed using cell cycle inhibitors, hydroxyurea and colcemid. Hydroxyurea is an inhibitor of ribonucleotide reductase to deplete dNTPs and stops the cell cycle in G_1 before DNA replication. Replication stress also inhibits

dephosphorylation of Cdc2 (de Vries *et al.*, 2005). In the case of BmN cells, hydroxyurea treatment induced accumulation of G_1 cells and phosphorylated Cdc2 (Fig. 2-2A and B). The antibody effectively detected the state of phosphorylation of *Bombyx* Cdc2. Furthermore, M phase arrest was archived by treatment of colcemid which was an inhibitor of microtubule polymerization (Fig. 2-2A). The colcemid treated cells underwent the chromatin condensation which showed M phase and active dephosphorylation of Cdc2 in M phase cells was confirmed (Fig. 2-2B). The expression level of total Cdc2 was unchanged by each inhibitor treatment.

The phosphorylation state of Cdc2 under high temperature stress condition was examined using the anti-phospho-Cdc2 antibody. Both of total and phosphorylated Cdc2 levels were constant at 26°C, while only the phosphorylation level was increased by incubation for 24 or 48 h at 38°C (Fig. 2-3).

Activation of p38 MAPK induced by high temperature

Western blotting with a specific antibody of phospho-p38 was performed for observation of high temperature effect on phosphorylation of p38. No phospho-p38 was detected in keeping with cultivation temperature at 26°C (Fig. 2-4A). On the other hand, shift in temperature to 38°C activated p38 phosphorylation after 2 h and sustained the level for 24 h. The phosphorylation at 38°C returned to the non-phosphorylated state by succeeding incubation at 26°C (Fig. 2-4B). Other

MAPK family proteins, ERK (extracellular signal-related kinase) and JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) were also examined the phosphorylation state at high temperature. ERK was phosphorylated by heat stress, as in the case of p38, but JNK was not (data not shown).

The effect of p38 inhibitor on cell cycle arrest by high temperature

BmN cells were exposed to a p38 inhibitor, SB202190 for 1 h at 26°C, and then subjected to high temperature. Although no proliferation of BmN cells was usually observed at 38°C, addition of SB202190 induced a little increase of cell number in the stress condition (Fig. 2-5A). Furthermore, cell cycle analysis using LSC showed that pretreatment of the cells with SB202190 inhibited heat-induced cell cycle arrest, decreasing the percentage of the cells in G_2/M phase from 46% to 41% at 5 μM and 42% at 10 μM (Fig. 2-5B). The high phosphorylation level of Cdc2 at 38°C was also suppressed by SB202190 treatment (Fig. 2-5C). There were no effect on the proliferation, cell cycle, and phosphorylated Cdc2 level of BmN cells pretreated with 5 or 10 μM SB202190 at 26°C.

Generation of intracellular ROS at high temperature

To examine the involvement of high temperature stress in generation of reactive oxygen species (ROS), observation with DCFH-DA, which is a specific dye for H_2O_2, was conducted. High

temperature treatment caused an increase in fluorescence which showed generation of ROS only for 2 h (Fig. 2-6A). More intense green fluorescence was detected in the cells incubated for 4 h under high temperature than moderate temperature. ROS continue to be generated by heat treatment for 24 h.

To confirm the specificity of this detection, antioxidant which catalyzed degradation of ROS was used. Although pretreatment with ascorbic acid decreased the intensity of the green color at 38°C, the effect of the antioxidant was disappeared only for 4 h and intense green fluorescence was detectable thereafter (Fig. 2-6B).

Involvement of ROS accumulation in cell cycle arrest

The addition of ascorbic acid delayed the accumulation of ROS. The effects of ascorbic acid treatment on activation of p38 and cell cycle arrest in G_2 phase under heat shock were investigated. Phosphorylation of p38 in the heat-treated cells was delayed about for 2 h by preincubation with ascorbic acid (Fig. 2-7A). Growth arrest by high temperature was a little recovered in ascorbic acid treated-cells (Fig. 2-7B). Analysis of cell cycle clarified that ratio of the cells in G_2/M phase at 38°C was reduced from 55% to 45% by addition of ascorbic acid (Fig. 2-7C). The cells at 26°C showed no response to ascorbic acid in the cell proliferation and cell cycle.

Direct effect of H_2O_2 on cell cycle was examined by addition of H_2O_2 to the medium. Exposure

to H_2O_2 for 30 min induced intense oxidative stress in the cells (Fig. 2-8A). The intense fluorescence was greatly reduced by pretreatment with ascorbic acid. Laser scanning analysis revealed that the oxidative stress arrested cell cycle in G_2 phase (Fig. 2-8B). The fraction of G_2 cells returned to the usual level by removal of H_2O_2 from the medium by changing the medium to the fresh one.

DISCUSSION

The mechanism of heat-induced cell cycle arrest

High temperature changes the expressions of many genes (Sørensen *et al.*, 2005; Zhou *et al.*, 2007). The reduction of cyclin B expression causes cell cycle arrest in G_2 phase in lepidopteran cell line, IAL-PID cells, treated with 20-hydroxyecdysone (Mottier *et al.*, 2004). In the case of BmN cells at high temperature, it was possible that the reduced expression of cell cycle genes involved in G_2/M transition induced the arrest of cell cycle. However, there was little difference in the expression levels of cdc2, cyclin A, cyclin B, and cdc25 between 26 and 38°C (Fig. 2-1). No difference was also observed in ribosomal protein 49, which is a housekeeping gene, and cyclin D, which is known as G_1 cyclin. These results suggest that the heat treatment for 24 h at 38°C unchanged the expression levels of most genes in BmN cells and the arrest of cell cycle under high temperature is not controlled by transcriptional level. On the other hand, the expression of heat shock protein 70 increased greatly by heat treatment in agreement with the case of fruit fly (Palter *et al.*, 1986).

Phosphorylation of Thr14 and Thy15 is particularly important in the control of Cdc2 activation at mitosis. To transit from G_2 to M phase, Thr14 and Thy15 are both dephosphorylated by a dual-specificity phosphatase termed Cdc25 (Morgan, 1995). Namely, cell cycle arrest in G_2 phase usually results from holding Cdc2 in an inactive phosphorylation state to leave Cdc25 from CDK

complex (Cyclin B-Cdc2). Therefore, I examined phosphorylation state of Cdc2 in the cells cultured at high temperature.

First, the cross-reactivity of anti human phospho-Tyr15-Cdc2 and Cdc2 (PSTAIRE) antibody to *Bombyx* proteins was checked using inhibitors of cell cycle. It was expected that anti human Cdc2 antibodies cross-reacted with *Bombyx* Cdc2, because the phosphorylation site of Cdc2 and the PSTAIRE sequence were nearly identical in amino acid sequences between human and silkworm. Treatment with hydroxyurea was used as a good positive control in high phosphorylated state of Cdc2. Hydroxyurea which inhibits DNA replication and arrest of cell cycle in G_1 is effective on insect cell lines (Siaussat *et al.*, 2004). In addition, hydroxyurea induces accumulation of inhibitory phosphorylated Cdc2 in *Drosophila* Schneider's cells (de Vries *et al.*, 2005). BmN cells treated with hydroxyurea also stopped the cell cycle at G_1 and accumulated phosphorylated Cdc2 (Fig. 2-2A and B). Furthermore, treatment with colcemid, which is an inhibitor of microtubule polymerization, was used as a good control in low phosphorylated state of Cdc2. Because of arresting the cell cycle in M phase, almost all Cdc2 is converted to active dephosphorylated form by colcemid treatment. In fact, colcemid was effective on BmN cells; nuclear aggregation which characterized M phase was observed in most cells and the level of phosphorylation decreased clearly. No effect of both cell cycle inhibitors on protein expression level of total Cdc2 was confirmed using anti human Cdc2 (PSTAIRE) antibody. These results show that phosphorylation state on *Bombyx* Cdc2 was detectable

by Western blotting using anti human Cdc2 antibodies.

The phosphorylation level of Cdc2 increased in BmN cells incubated at high temperature (Fig. 2-3). This result suggests that the inactivation of Cdc2 is important process for heat-induced cell cycle arrest in G_2 phase. Stress-activated p38 MAPK phosphorylates Cdc25 and activates G_2/M checkpoint pathway in response to various environmental insults, including UV radiation and hyperosmotic stress (Bulavin *et al.*, 2001; Smith *et al.*, 2002; Lopez-Aviles *et al.*, 2005; Clotet *et al.*, 2006). Besides these stresses, heat shock and oxidative stress also activate p38 MAPK in mammalian cells, fission yeast, fruit fly, and the cell lines (Degols *et al.*, 1996; Han *et al.*, 1998; Adachi-Yamada *et al.*, 1999; Zhou *et al.*, 2005; Aggeli *et al.*, 2006; Zhuang *et al.*, 2006; Kani *et al.*, 2007). These reports suggest that G_2/M arrest by high temperature is related to signal pathway mediated by p38 activation and following Cdc2 inactivation.

In fact, High temperature activated p38 in BmN cells as the case of *Drosophila* cell lines (Fig. 2-4A). Heat-induced phosphorylation returned to non-phosphorylated state by decrease of temperature from 38 to 26°C (Fig. 2-4B). This result corresponds to reversibility of G_2/M arrest at high temperature (CHAPTER 1). The arrest of cell cycle and proliferation under heat stress condition were a little rescued by addition of p38 inhibitor, SB202190, into the medium (Fig. 2-5A and B). Furthermore, the accumulation of phosphorylated Cdc2 at high temperature was also declined by SB202190 treatment (Fig. 2-5C), indicating that high temperature stress arrested the cell

cycle in G_2 phase by inactivation of Cdc2 mediated by p38 activation. However, treatment with SB202190 was not able to recover completely the arrest. The following three possibilities are under consideration for this incomplete recovery; specificity of the inhibitor, relationship of another pathway, and the necessity of p38.

Four isoforms are known as human p38 MAPK (p38α, β, γ, and δ). SB202190 preferentially targets the p38α and p38β, but does not p38γ and p38δ (Nebreda and Porras, 2000). *Bombyx* p38 are more similar to p38α and p38β than p38γ or p38δ, and SB202190 is expected to have inhibitory effect on *Bombyx* p38 for this reason. However, there remains a possibility that the specificity of SB202190 is insufficient for *Bombyx* p38. Knockdown of p38 using RNA interference would be a useful tool to clarify this possibility.

It is likely that the some signal pathways coordinate in G_2/M arrest by heat stress. Miyakoda *et al.* (2002) observed that heat shock induces ataxia-telangiectasia mutated (ATM) activation in mammalian cell lines. DNA damage checkpoint caused by UV radiation, ionizing radiation and others are well studied as the mechanism of G_2/M arrest involved in control of Cdc2 phosphorylation state (Sanchez *et al.*, 1997; de Vries *et al.*, 2005). Damaged DNA is sensed by ATM and ATR (ATM and Rad3-related) kinase families. These kinases transmit the signal to two downstream kinases, Chk1 and Chk2. Cdc25 phosphatase is phosphorylated by Chk1 and Chk2, and then bind to 14-3-3 proteins which are nuclear export factors. Separation of Cdc25 from CDK complex in nucleus

inhibits Cdc2 activation and arrests the cell cycle in G_2 phase (Lopez-Girona *et al.*, 1999). The participations of ATM/ATR and the downstream proteins Chk1/Chk2 in the G_2/M arrest at high temperature are considerable. Inhibition of p38 MAPK only may be insufficient to recover completely G_2/M arrest at high temperature. Although, high temperature stress also phosphorylated another MAPK protein ERK, U0126 which is an inhibitor of ERK phosphorylation could not recover the arrest of cell cycle and proliferation in BmN cells under high temperature (unpublished data). From this result, I infer that ERK phosphorylation is independent of the G_2/M arrest by heat stress.

The last possibility is that the p38 activation is needed for heat tolerance of BmN cells. *Drosophila* lacking p38 are susceptible to some environmental stress, including heat shock, oxidative stress and starvation (Craig *et al.*, 2004). There are some reports that induction of heat shock proteins is involved in p38 activation in mammalian cell (Uehara *et al.*, 1999; Rafiee *et al.*, 2006). These results suggest the possibility that more serious damages than G_2/M arrest occurred in the cells by the inhibition of p38 MAPK at high temperature.

Heat-induced ROS generation and the involvement in the cell cycle arrest

What occurs in the heat-treated cells? Our study discovered the accumulation of reactive oxygen species (ROS) in the cells incubated at high temperature (Fig. 2-6). There are close relationship

between high temperature stress and oxidative stress. The expressions of heat shock proteins are also induced when the cells are exposed by oxidative stress (Courgeon *et al.*, 1988, 1990; Jornot *et al.*, 1991). On the other hand, overexpression of antioxidative genes in yeast provides heat resistance (Davidson *et al.*, 1996). High temperature induces ROS accumulation in many species (Kim *et al.*, 2006; Volkov *et al.*, 2006). In the silkworm, the expression of an antioxidant enzymes, thioredoxin peroxidase, is induced in transcriptional and translational level by heat shock (Lee *et al.*, 2005; Kim *et al.*, 2007), suggesting that ROS is accumulated under high temperature in the silkworms. Generation of ROS probably damages proteins, lipids, and particularly DNA in BmN cells. These damages cause activation of p38, and then the signal is transmitted to G_2/M checkpoint pathway. Recently, two reports show the evidence that heat shock induces double-strand breaks in mammalian cells (Takahashi *et al.*, 2004; Kaneko *et al.*, 2005). If the double-strand breaks occurred in BmN cells at 38°C, involvement of pathways mediated by ATM and ATR remain controversial.

To prevent ROS accumulation by heat stress, ascorbic acid which is one of antioxidants was added in the medium. Addition of ascorbic acid decreased generation of ROS in the normal state and delayed induction of the heat stress state (Fig. 2-6). Together with the retardation of ROS accumulation, the phosphorylation of p38 by high temperature was delayed and weakened (Fig. 2-7A). Furthermore, pretreatment with ascorbic acid recovered slightly the cell growth and cell cycle arrest at high temperature (Fig. 2-7B and C). On the other hand, BmN cells directly treated with

H_2O_2 (one of the ROS) showed strong oxidation and a series of G_2/M arrest similar to the observation under heat stress condition (Fig. 2-8A and B). From these results, I propose that abnormal high temperature for insects induces oxidative damage to the cells and consequently activates G_2/M checkpoint pathway. However, my study is not capable for denying the possibility that high temperature damages to the cells directly. Treatment with ascorbic acid is insufficient to prevent BmN cells from accumulating ROS. If ROS generation was completely suppressed by treatment with other antioxidants or overexpression of antioxidant enzymes, the question would be solved.

FIGURES AND TABLES

Name	Sequence (5' to 3')
B.mori cdc2 F	GACGCTTACATCAGAGTGAC
B.mori cdc2 R	GGAACCCAACTGAGTTGTAC
B.mori cyclin A F	TGCACAGACCGACAGAGA
B.mori cyclin A R	CTGTTCCGACAAGTTGAAGC
B.mori cyclin B F	GCCGGAGACAACAACAGC
B.mori cyclin B R	GGGTCTCGCAAGACAGAA
B.mori cdc25 F	AACTGATCCGAGGAGCTTAC
B.mori cdc25 R	CGTTCCAATGAGAATTCGCAG
B.mori cyclin D F	AGTGGATGCTAGAGGTATG
B.mori cyclin D R	CAACAGTAGACAGGCTGTG
B.mori rp49 F	TCGGATCGCTATGACAAAC
B.mori rp49 R	ACTAGGACCTTACGGAATC
B.mori hsp70 F	GGCAACCGTATCACACCATC
B.mori hsp70 R	TTCTTCTGGCGCAAATCGTT

Table 2-1

Oligonucleotide primers used in the real-time quantitative PCR.

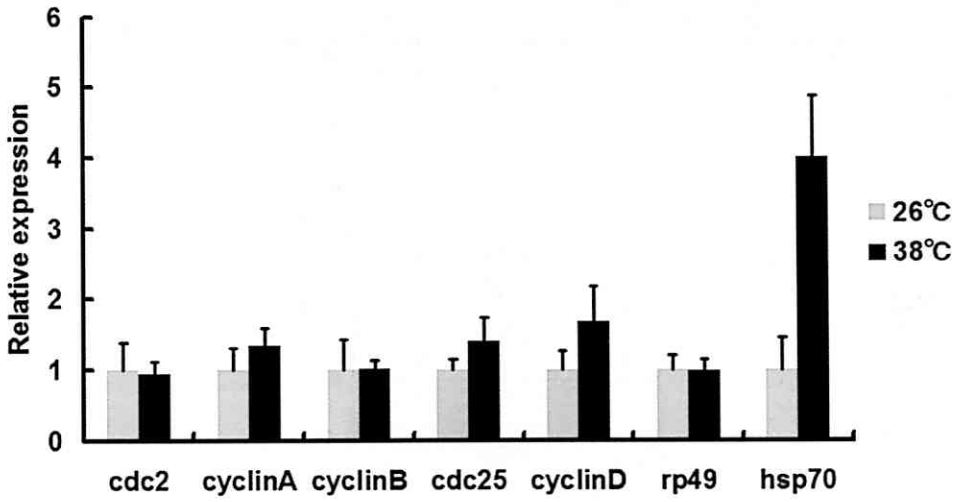

Figure 2-1

Expression pattern of cell cycle genes. BmN cells were incubated for 24 h at 26 or 38°C and then total RNA were isolated. The RNA was converted to cDNA and amplified by real-time quantitative PCR. The level of each cell cycle gene is means of three assays, which is calculated relative to that of the expression recorded for the level at 26°C. Error bars, SD.

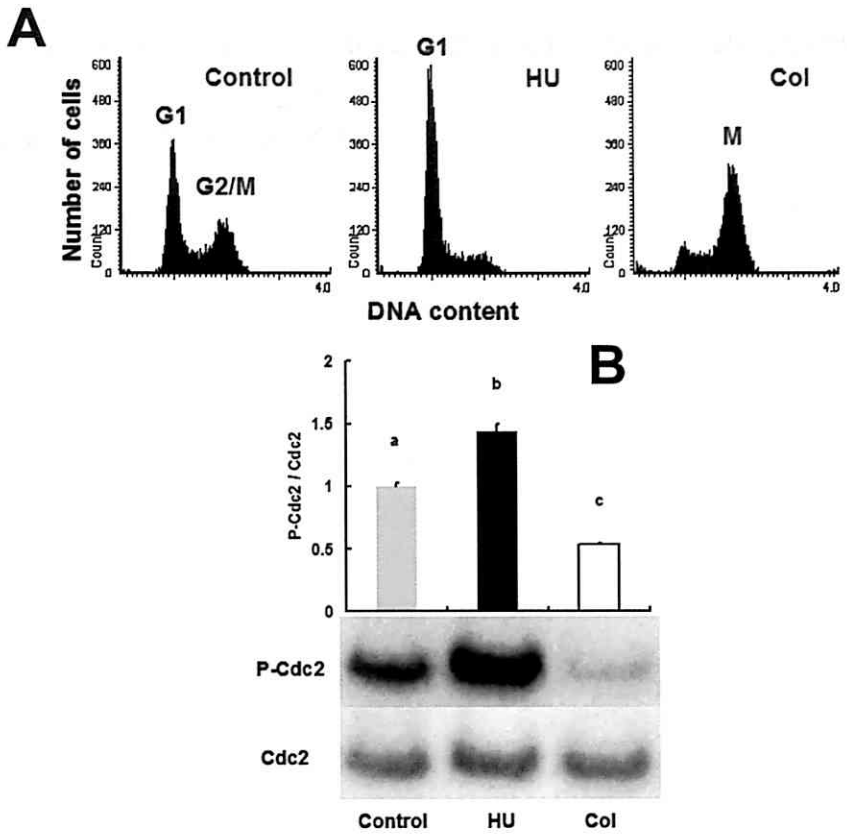

Figure 2-2

The effect of cell cycle inhibitors on BmN cells. (A) BmN cells treated with 1 mM hydroxyurea (HU) or 0.25 μg/ml colcemid (Col) were incubated for 48 h at 26°C and then fixed. After the DNA stained with propidium iodide, content and intensity of fluorescence was measured by LSC. The histograms were drawn using LSC software. The phase of cell cycle is indicated with each peak. G_2 and M phase were discriminated by fluorescence intensity which reflected chromatin condensation. (B) Phospho-Cdc2 or total-Cdc2 levels of BmN cells treated with hydroxyurea or colcemid for 48 h were detected by Western blotting using antibodies against phospho-Tyr15-Cdc2 or Cdc2 (PSTAIRE). The blot presented is representative of three separate experiments. Blots were analyzed by Scion Images. The relative densitometric readings (control = 1) of P-Cdc2/Cdc2 from three

separate experiments are presented in the upper panel as means ± SD. Bars with different letters indicate statistical difference at $P < 0.01$.

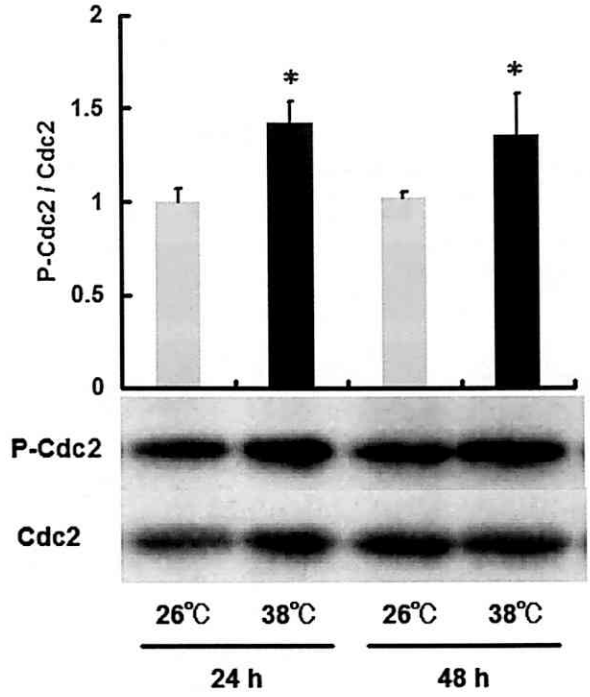

Figure 2-3

The effect of high temperature on Cdc2 phosphorylation. BmN cells were cultured for indicated time at 26 or 38°C. The cells were then dissolved in sample buffer and analyzed by Western blotting using antibodies against phospho-Tyr15-Cdc2 or Cdc2 (PSTAIRE). The blot presented is representative of three separate experiments. Blots were analyzed by Scion Images. The relative densitometric readings (control = 1) of P-Cdc2/Cdc2 from three separate experiments are presented in the upper panel as means ± SD. Bars with different letters indicate statistical difference at $P <$ 0.05.

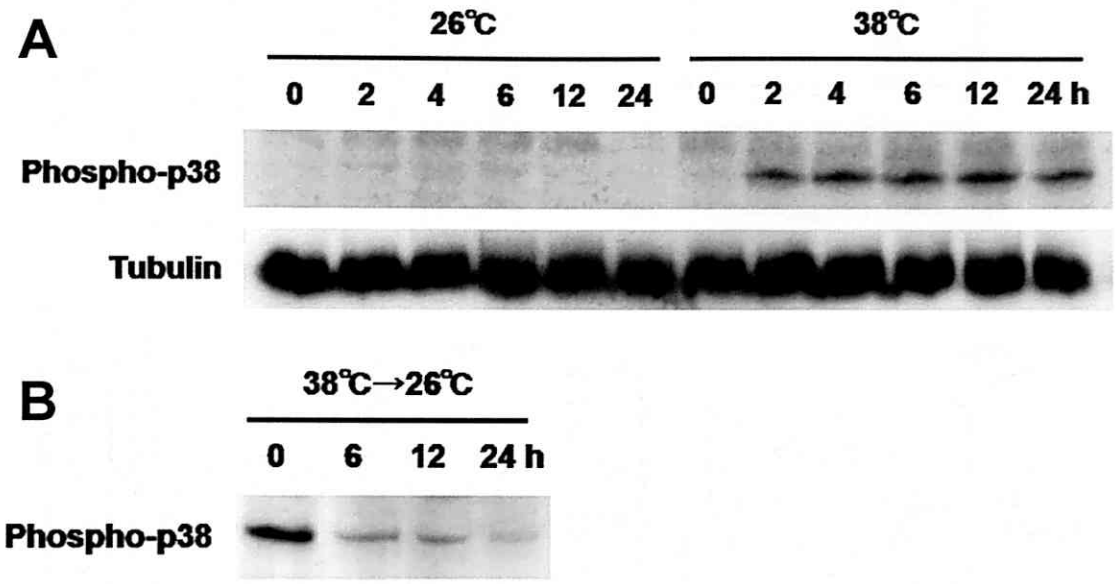

Figure 2-4

The effect of high temperature on p38 phosphorylation. (A) BmN cells were cultured for indicated time at 26 or 38°C. The cells were then dissolved in sample buffer and analyzed by Western blotting using antibody against phospho-p38. Tubulin blotting shows equal loading. (B) BmN cells incubated for 24 h at 38°C were shifted in temperature to 26°C and subsequently incubated for indicated time.

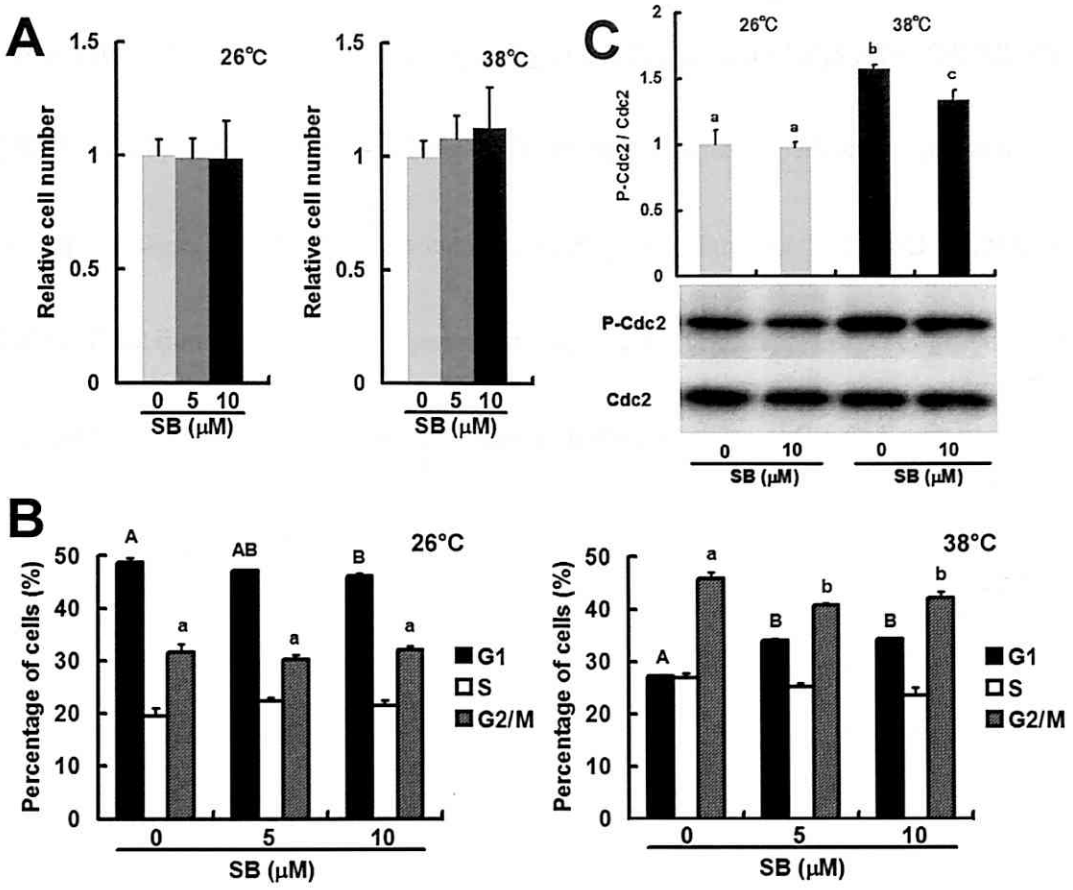

Figure 2-5

The effects of p38 inhibitor on heat-induced cell cycle arrest. (A) BmN cells were pretreated with SB202190 (SB) for 1 h and then cultured at 26 or 38°C for 24 h. The cell numbers were then counted under an inverted microscope. The relative cell numbers (no inhibitor in DMSO = 1) from three independent wells were indicated as means ± SD. (B) The cells pretreated with SB202190 were incubated for 24 h at 26 or 38°C, and then fixed. Fixative cells were stained with DNA dye (propidium iodide) and analyzed the cell cycle using LSC. Data represent the means ± SD of three independent experiments and different letters indicate statistical difference at $P < 0.05$. (C) The cells pretreated with SB202190 cultured for 24 h at 26 or 38°C. The cells were then dissolved in sample

buffer and analyzed by Western blotting using antibodies against phospho-Tyr15-Cdc2 and Cdc2 (PSTAIRE). The blot presented is representative of three separate experiments. Blots were analyzed by Scion Images. The relative densitometric readings (no inhibitor in DMSO at 26°C = 1) of P-Cdc2/Cdc2 from three separate experiments are presented in the upper panel as means ± SD. Bars with different letters indicate statistical difference at $P < 0.05$.

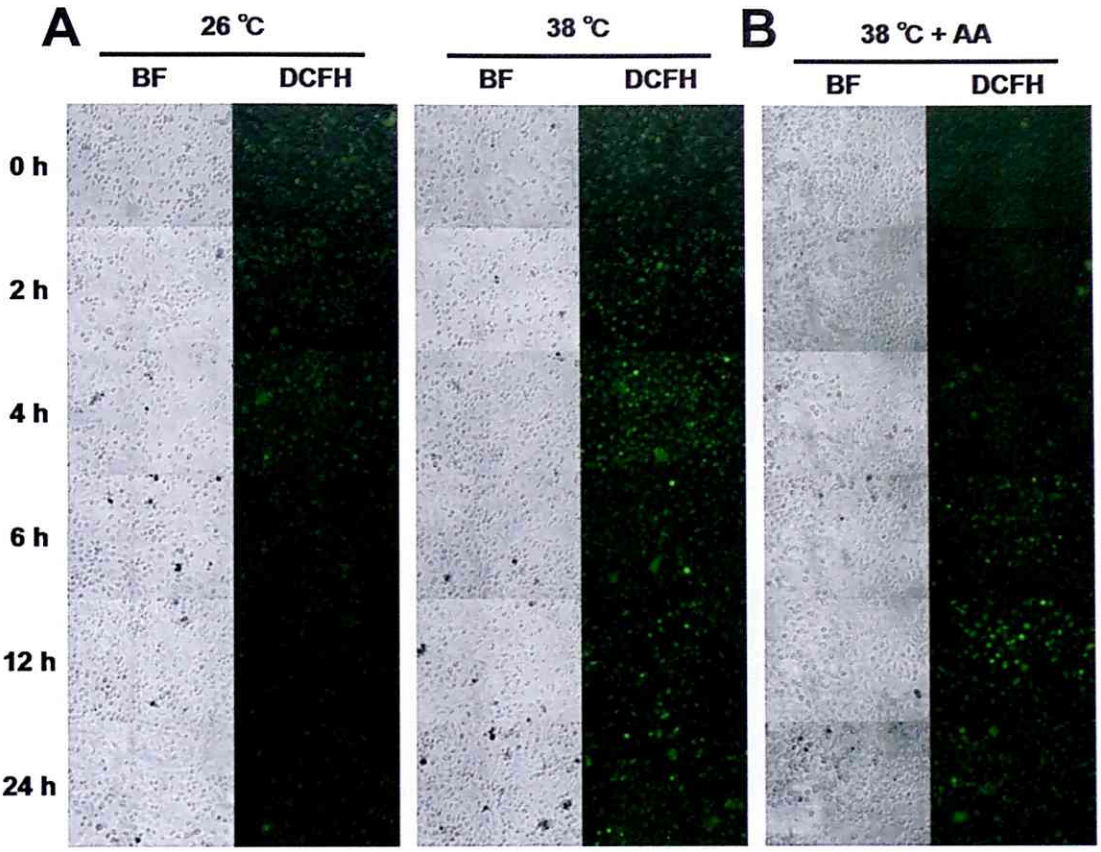

Figure 2-6

Generation of ROS by high temperature stress. (A) BmN cells were cultured for indicated times at 26 or 38°C, and then treated with 50 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA; Specific dye for H_2O_2). After incubated with DCFH-DA for 30 min at 26°C, the cells were washed three times with insect PBS and immediately analyzed using fluorescent microscopy. BF: Blight field, DCFH: Green channel. (B) BmN cells were preincubated with 500 μM ascorbic acid (AA) for 24 h at 26°C. Ascorbic acid-treated cells were shifted in temperature to 38°C and cultured for indicated time.

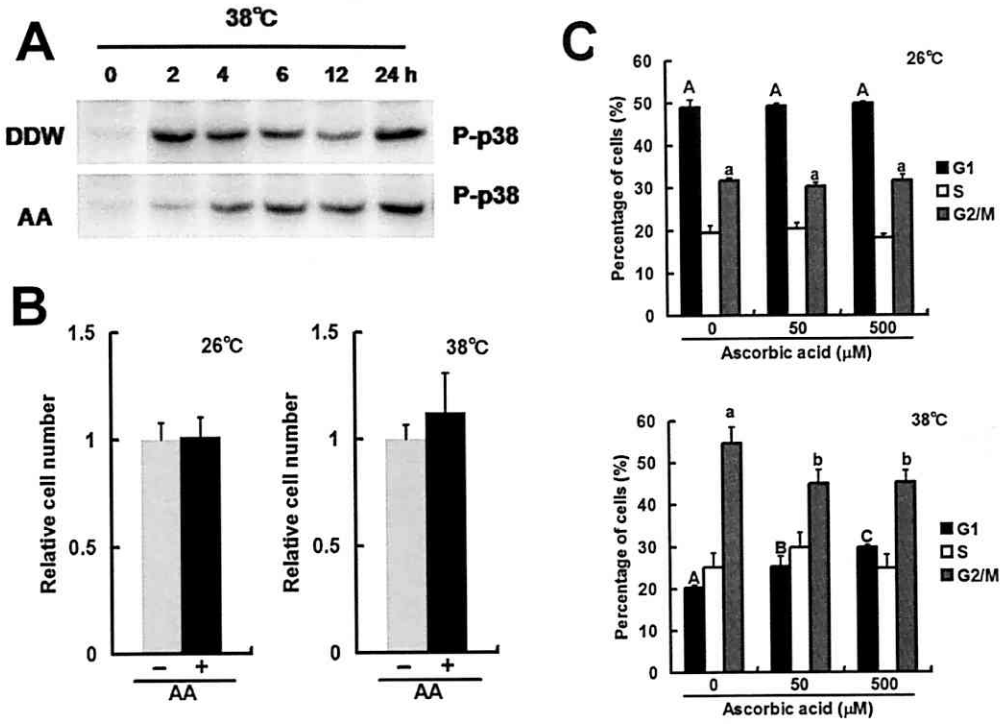

Figure 2-7

The effects of ascorbic acid on the cell cycle arrest mediated by heat activated p38. (A) BmN cells were incubated with or without 500 μM ascorbic acid (AA) for 24 h at 26°C. Then, the cells were shifted in temperature to 38°C. The cells were dissolved in sample buffer at indicated time and analyzed by Western blotting using antibody against phospho-p38. (B) BmN cells pretreated with ascorbic acid were cultured for 24 h at 26 or 38°C and then counted under an inverted microscope. The relative cell numbers (no antioxidant in DDW = 1) from three independent wells were indicated as means ± SD. (C) The cells pretreated with ascorbic acid were cultivated for 24 h at 26 or 38°C and then laser scanning cytometry was performed. Data represent the means ± SD of three

independent experiments and different letters indicate statistical difference at $P < 0.05$.

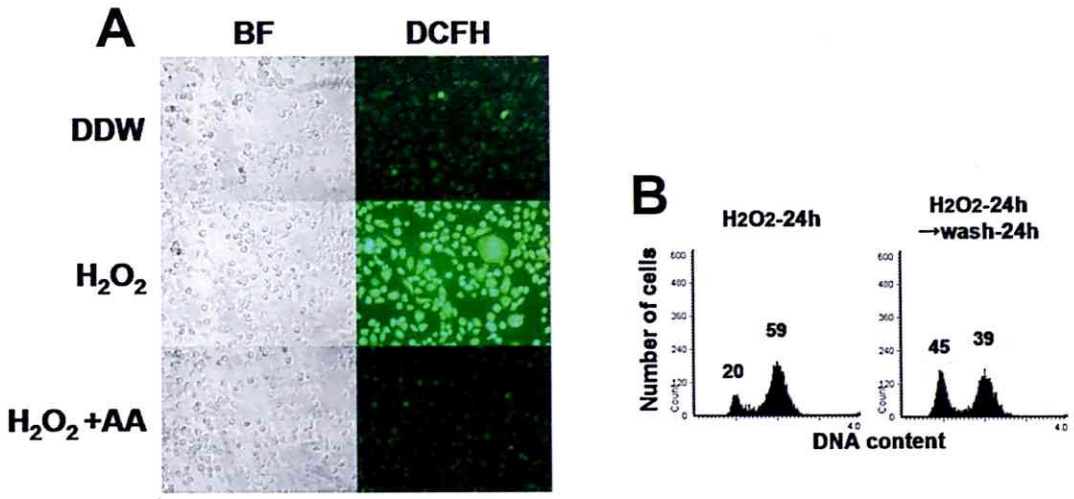

Figure 2-8

The effects of H_2O_2 on BmN cells. (A) BmN cells were incubated with DCFH-DA (final 50 μM) for 1 h, and subsequently treated with 100 μM H_2O_2 for 30 min at 26°C. After the treatment, the cells were washed three times with insect PBS and immediately analyzed using fluorescent microscopy. To examine the effects of antioxidant, BmN cells were preincubated with ascorbic acid (AA) for 24 h at 26°C. BF: Blight field, DCFH: Green channel. (B) Cell cycle analysis using LSC were attempted in the cells incubated with 100 μM H_2O_2 for 24 h at 26°C. The recovery from the arrest of cell cycle was examined when H_2O_2 were removed from the medium by washing. The washed cells were subsequently incubated for 24 h and then laser scanning cytometry was performed. The

histograms were drawn using LSC software. The percentages of cell cycle in G_1 and G_2/M phase are indicated above each peak.

CHAPTER 3

Effects of High Temperature on the Hemocyte Cell Cycle in Silkworm Larvae

INTRODUCTION

A close relationship exists between insect development and temperature. In a number of insect species, the growth rate is increased and the developmental period is shortened as the temperature increases (Wigglesworth, 1972). However, each species has its own range of optimal temperatures for growth, and extremely high temperatures inhibit rather than promote growth by inducing growth delays and developmental failures, such as larval ecdysis and adult emergence (Chapman, 1998). The mechanisms underlying these effects are unknown. In this chapter, I sought to understand the effects of high temperature on insect growth at the cellular level.

I investigated the effects of high temperatures on cultured cells derived from the silkworm, *Bombyx mori* (CHAPTER 1). At 38°C, which is an unusually high temperature for silkworms, the cells did not die but were arrested in G_2. From this result, I speculated that the induction of a cell cycle arrest by high temperatures is closely related to the suppression of insect growth and development. To confirm this speculation, I investigated whether the cell cycle arrest observed in

cultured cells in response to high temperatures also occurs in silkworms.

Cell division in insects depends on the developmental stage of the individual and is strongly regulated by the hemolymph concentration of such hormones as 20-hydroxyecdysone (20E) and juvenile hormone (JH). Gardiner and Strand (2000) showed that lepidopteran hemocyte growth tended to be higher at the feeding stage (i.e., the middle of each instar) than at the molting stage. Koyama *et al.* (2004) demonstrated the control exerted by 20E on the cell cycle in *Bombyx* wing discs in detail, while Truman *et al.* (2006) showed that JH suppresses the growth and development of imaginal tissues in starved *Manduca* larvae after ecdysis. These results suggest that cell cycle varies over the course of development in response to endocrine signals. For these reasons, the status of the cell cycle during each growth phase must be understood in order to analyze the effects of high temperatures on insect cells.

It is difficult to monitor the progression of insect cells through the cell cycle based on DNA content, because endoreplication (i.e., the replication of DNA during S phase without the subsequent completion of mitosis) is a widespread phenomenon in arthropods, meaning that many insect cells are polyploid (Smith and Orr-Weaver, 1991; Edgar and Orr-Weaver, 2001). This poses a very serious problem for cell cycle analysis, since the DNA content of a diploid cell at G_2 is equal to that of tetraploid cell at G_1.

In this chapter, cell proliferation and division were studied using *Bombyx* hemocytes, which are

easily isolated. Silkworm hemocytes are classified into five morphotypes: granulocytes, plasmatocytes, prohemocytes, spherulocytes, and oenocytoids (Nittono, 1960; Akai and Sato, 1973; Wago, 1991). Larval hemocytes are produced in the hematopoietic organ (Akai and Sato, 1971), and proliferate by mitosis in the hemolymph (Arnold and Hinks, 1976, 1982; Beaulaton, 1979; Feir, 1979; Gardiner and Strand, 2000). Mitosis has been observed in prohemocytes, granulocytes, and, rarely, in spherulocytes (Nittono, 1960). The number of hemocytes and their components fluctuates during growth (Gardiner and Strand, 2000).

Here, I investigated hemocyte proliferation and division in *Bombyx mori* through the larval molting cycle, and I assessed the effects of high temperatures on these events. To understand the effects of temperature on the hemocyte cell cycle, I began by characterizing hemocyte proliferation and division at each stage of larval development under normal conditions. I then analyzed the DNA content of each type of hemocyte and its progression through the cell cycle.

MATERIALS AND METHODS

Silkworms

Shoon *Bombyx mori* silkworms were used in this study. The larvae were reared on an artificial diet (Nihonnosankogyo Co., Japan) under a continuous cycle of 12 h light and 12 h darkness at 25 ± 2°C until the third instar. Twenty newly ecdysed larvae in the fourth instar were then transferred to a plastic case (20.5 cm × 15.0 cm × 5.2 cm) and reared at 26 or 38°C without light. Fluctuations in temperature in the incubators were controlled within 0.5°C and the humidity was maintained at 60-70%. Fresh food was administered each day to avoid deterioration, and the weight of each larva (three males and three females) was measured until ecdysis to the fifth instar.

Hemocyte quantitation

Hemolymph was collected onto Parafilm™ from incisions made in the caudal horn of each larva, and 5 μl of the fluid were placed immediately into a single well of a 96-well microplate with 100 μl of phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) containing 10% formalin and 0.001% 1-phenyl-2-thiourea to prevent melanization of the hemolymph. The hemocytes, which were fixed at the bottom of the well, were then counted (five random areas, 6.35×10^{-4} cm^2 each) under an inverted microscope, and the density of the hemocytes was calculated. Our preliminary results showed that almost all of the hemocytes were fixed at the

bottom. The amount of hemolymph contained in a fourth-instar larva is estimated at 30% of the total body weight (Nagata *et al.*, 1980). Thus, the total number of hemocytes per larva was estimated by multiplying the hemocyte density by the estimated hemolymph volume.

Detection of mitotic hemocytes

The number of hemocytes undergoing mitosis was assessed immunohistochemically as described by Champlin and Truman (1998) and Koyama *et al.* (2004) with minor modifications. Hemolymph was collected from the larvae (three males and three females) by the above-mentioned method and mixed by gently pipetting on Parafilm™. A 50-100 µl aliquot of the mixture was then placed on the 1 × 1-cm^2 area of a slide coated with poly-Lysine (POLY-PREP™ SLIDES; Sigma-Aldrich Co., Ltd., Dorset, UK) and 0.001% 1-phenyl-2-thiourea was added. The hemocytes were allowed to attach to the slides at room temperature for 10 min; subsequently, they were fixed for 10 min in PBS containing 3.7% paraformaldehyde. After three washes in Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) for 5 min each, the hemocytes were permeabilized in TBS containing 0.1% Triton X-100 for 5 min, rinsed in TBS, and blocked for 15 min in 1% bovine serum albumin-solubilized TBS. The hemocytes were then rinsed in TBS for 5 min and immunostained with a 1:200 dilution of anti-phospho-histone-H3 (Ser10) antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 24 h at 4°C. After being washed three times in TBS for 5 min each, the

hemocytes were incubated with a 1:400 dilution of FITC-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. After immunostaining, 100 µl of a solution containing 50 µg/ml RNase (Ribonuclease; Roche Diagnostics Co., Indianapolis, IN, USA) and 5 µg/ml propidium iodide (PI; Sigma, St. Louis, MO, USA) were added to the hemocytes and incubated for 1 h at 37°C. Finally, the hemocytes were washed three times in TBS for 5 min each and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). The immunostained hemocytes were observed by fluorescence microscopy using a UV excitation filter (BX51, Olympus, Tokyo, Japan) and photographed with a CCD camera (DP70, Olympus).

Cell cycle analysis and classification of the hemocytes

Fixed hemocytes were washed three times with 100 µl of PBS; 100 µl of RNase-PI (50 µg/ml RNase and 5 µg/ml PI) were then added, and the cells were incubated for 24 h at 37°C. Finally, the hemocytes were mounted in Vectashield, and cellular fluorescence was measured with a laser scanning cytometer (LSC; Olympus; Luther and Kamentsky, 1996; Darzynkiewicz *et al.*, 1999). PI fluorescence (610-620 nm) was detected using an argon laser at 488 nm. An average of 10,000 hemocytes per slide was scanned automatically using a 40× objective lens. At the start of scanning for each slide, the threshold of photomultiplier tube (PMT) sensitivity was adjusted such that

maximum detection of the stained areas was achieved with minimal detection of background staining. The silkworm hemocytes were classified into five types based on the criteria of Nittono (1960) and Akai and Sato (1971, 1973).

Western blotting

Hemolymph were collected into a 1.5 ml tube on ice and centrifuged at 4,000 rpm for 10 min at 4°C. Precipitated hemocytes were rinsed with cold PBS, then lysed with 30 μl of SDS-sample buffer (50 mM Tris-HCl: pH 6.8, 2% sodium dodecyl sulfate, 0.86 M 2-mercaptoethanol, 10% glycerol). After the samples denatured by boiling, the protein amount was measured by using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was conducted by the method of Laemmli (Laemmli, 1970). Twenty five μg of proteins were analyzed in 10% polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA, USA) using transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The membranes were blocked for 1 h at room temperature in TBS/T buffer (0.1% Tween-20, 25 mM Tris, and 150 mM NaCl, pH 7.6) containing 2% ECL Advance blocking agent. The blotted membranes were washed three times for 5 min each with TBS/T buffer and then incubated overnight at 4°C with each primary antibody. Used antibodies were rabbit anti-phospho-Cdc2 (Sigma) and anti-Cdc2 (PSTAIRE, Upstate Biotechnology). They were diluted at 1:2000 in TBS/T containing 5% bovine serum

albumin. After incubation with the primary antibodies, the membranes were washed five times with TBS/T. Secondary antibodies, horse radish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology Inc., Beverly, MA, USA) diluted at 1:4000 in TBS/T buffer containing 2% ECL Advance blocking agent, were then added and further incubated for 1 h at room temperature. The membranes were then washed five times in TBS/T. Immunoreactivity was visualized using an ECL Advance Western Blotting Detection Kit (Amersham Biotech, Little Chalfont Buckinghamshire, UK) and LAS-1000 Luminescence Image Analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). Densitometric analyses were conducted through use of a PC software, Scion Images for Windows (Scion Co., Frederick, MD, USA).

RESULTS

Effect of high temperature on hemocyte proliferation

To estimate the number of circulating hemocytes in fourth-instar larvae reared at 26 or 38°C, larval body weight and hemocyte density in the hemolymph were examined. At 26°C, body weight increased constantly from day 0 to day 3 (Fig. 3-1A). When the weight of the larvae reached about 0.8 g, the larvae stopped feeding and entered the molting phase. Head capsule slippage, which signals the beginning of the molting process, was observed between day 3 and day 4, and ecdysis to fifth-instar larvae occurred between day 4 and day 5. In contrast, at 38°C, little weight gain was observed up to day 4. The silkworms did not feed as often, and they gradually weakened and died without entering the molting phase. Hemocyte density ranged from approximately 3×10^6 to 5×10^6 cells/ml during the fourth larval stadium at 26°C; however, at 38°C, the density decreased to 1×10^6 cells/ml over 24 h and remained unchanged during the next 3 days (Fig. 3-1B). The number of circulating hemocytes in the larvae, calculated by multiplying the hemocyte density by the estimated hemocyte volume, is shown in Figure 3-1C. At 26°C, the total number of hemocytes in the larvae increased steadily from approximately 1×10^5 on day 0 to 10×10^5 cells on day 3, and then stopped at the molting phase. In contrast, during the same 4-day period, the hemocytes of the larvae kept at 38°C did not proliferate.

Effect of a temperature shift on hemocyte proliferation

Beginning at the fourth larval stadium, the rearing temperature was shifted from 26 to 38°C or from 38 to 26°C every day, and the number of hemocytes in each individual was counted. A shift in temperature from low to high on day 1 and day 2 resulted in a decrease in the weight gain compared to no shift in temperature (Fig. 3-2A). Body weight was also reduced by a high temperature shift on day 3 because the silkworms entered the molting phase. The higher temperature inhibited hemocyte proliferation, and the number of hemocytes subsequently decreased (Fig. 3-2B). When larvae reared at 38°C were transferred to 26°C, little recovery in body weight was observed; however, hemocyte proliferation quickly recovered to the level seen in larvae reared at 26°C for 1 day (Fig. 3-2C).

Effect of high temperature on hemocyte division

Immunostaining with an antibody against phospho-histone-H3 (an M phase marker) was used to examine the proportion of mitotic hemocytes. The ratio of mitotic hemocytes is illustrated in Figure 3-3A and B. Fourth-instar larvae just after ecdysis had few mitotic hemocytes in their hemolymph. Mitotic hemocytes were observed more frequently between days 1 and 2 than during any other period at 26°C. On days 3 and 4, when the silkworms stopped feeding and entered the molting phase, the number of hemocytes undergoing mitosis decreased again. A few hemocytes in metaphase were observed on days 1 and 2, but only rarely during the other stages. At 38°C, there were scarcely any

mitotic hemocytes in the fourth larval stadium, regardless of the day. Almost all of the mitotic hemocytes were granulocytes; mitotic prohemocytes and spherulocytes were infrequently observed, and no mitotic plasmatocytes or oenocytoids were found.

Effect of high temperature on the hemocyte cell cycle

The cell cycle progression of hemocytes isolated from silkworms was analyzed (Fig. 3-4 and Table 3-1). The hemocytes exhibited three main peaks of DNA content: 2C, 4C, and 8C. DNA content was defined in relation to that of a spermatogonium (1C). Although hemocytes with a DNA content greater than 16C existed, they were rare. Hemocytes with 2C (45.2%) and 4C (38.1%) nuclei were predominant, while those with 8C (16.7%) were in the minority at the beginning of the fourth larval stadium. The percentage of 2C hemocytes decreased gradually to 31.3% at IV3 (i.e., day 3 of the fourth larval stage) at 26°C, whereas the percentages of 4C and 8C hemocytes increased to 42.6 and 26.1%, respectively. After the silkworms entered the molting phase (i.e., at IV4), the percentage of 8C hemocytes dropped to 14.4% (2C: 42.8%, 4C: 42.8%) and the distribution of DNA content was similar to that at IV0. No significant difference was observed between IV0 (2C: 48.4%, 4C: 38.2%, and 8C: 13.4%) and IV1 (2C: 45.5%, 4C: 34.5%, and 8C: 20.0%) at 38°C. Among the silkworms reared at 38°C, the number of hemocytes with a higher DNA content increased steadily. At IV3, the percentages of 2C, 4C, and 8C hemocytes were 17.7%, 36.0%, and 46.3%, respectively.

A down-shift in DNA content at IV3 was not observed at 38°C.

Cell cycle analysis

To determine the types of hemocytes with each DNA content, I investigated the cell cycle status of each individual hemocyte type. After completing my analysis of the entire cell cycle, the hemocytes on each slide glass were classified into five morphotypes as per the criteria described in the MATERIALS AND METHODS. Representative photographs of each type of *Bombyx* hemocyte are shown in Figure 3-5A, and the proportion of hemocytes with 2C-8C in fourth-instar larvae is displayed in Figure 3-5B. Most of the cells were granulocytes at all stadia (over 50%) at 26°C; however, at 38°C, the number of granulocytes gradually decreased to 40%. The ratio of plasmatocytes increased to 32% on day 3 and then decreased during the molting phase (day 4) at 26°C; at 38°C, the ratio increased to 48% by day 3. The number of spherulocytes at the early phase in fourth-instar larvae was larger than at the late stage at both 26 and 38°C. The number of prohemocytes and oenocytoids remained extremely small at both 26 and 38°C.

Among the larvae grown at 26°C, the percentage of granulocytes with 2C nuclei decreased at IV1 and increased at IV4 (Fig. 3-5C); conversely, the percentage of 4C and 8C granulocytes increased at IV1 and decreased at IV4. At 38°C, the percentage of 8C granulocytes increased from 7 to 28% within 3 days, while the percentage of 2C granulocytes dropped from 58 to 35%. In the case of the

plasmatocytes, the percentages of 2C, 4C, and 8C cells were roughly equal in fourth-instar larvae before feeding (Fig. 3-5D); however, the DNA content of the plasmatocytes increased toward IV3 at 26 and 38°C. The percentage of 8C plasmatocytes decreased at IV4 after ecdysis. Almost all of the spherulocytes had a 2C DNA content, and they remained largely unchanged in fourth-instar larvae at 26 and 38°C (Fig. 3-5E). The status of the prohemocytes and oenocytoids was not determined because so few of each cell type were present.

Phosphorylation level of Cdc2

For proceeding from G_2 to M phase, Thr14 and Tyr15 on Cdc2 are both dephosphorylated by a dual-specificity phosphatase termed Cdc25 (Morgan, 1995). To examine the phosphorylation state of Cdc2 in the hemocytes of silkworm reared at high temperature, Western blotting using anti phospho-Tyr15-Cdc2 antibody was performed. Total amount of Cdc2 fluctuated through fourth-instar larvae at 26°C (Fig. 3-6A). Expression level of hemocyte Cdc2 was low at day 0 of the fourth larval stage. The expression level increased greatly on day 1 and reached the maximum on day 2. After that, Cdc2 decreased gradually between days 3 and 4. The fluctuation pattern of phosphorylated Cdc2 was similar to that of total-Cdc2 (Fig. 3-6B), namely the phosphorylation level of Cdc2 was largely invariable through the fourth larval stage (Fig. 3-6C). At 38°C, a little increase of Cdc2 was observed up to day 3. Because the increase of phospho-Cdc2 was higher than that of

total-Cdc2, the phosphorylation level of Cdc2 in the larvae at 38°C was 1.5 times as high as at 26°C.

DISCUSSION

Timing of hemocyte proliferation

Few hemocytes underwent mitosis until feeding (Fig. 3-3). When the silkworms stopped feeding and entered the molting phase, hemocyte proliferation was suppressed again. Our results are consistent with the pattern of hemocyte DNA synthesis in *Pseudoplusia includens* and *Spodoptera frugiperda* (Gardiner and Strand, 2000). In these lepidopterans, hemocyte growth is higher during feeding than molting. This pattern is similar to previously reported mitotic indices (Crossely, 1975; Pathak, 1986), suggesting that lepidopteran hemocyte proliferation fluctuates with the molting cycle. I confirmed the same pattern of cell division in *Bombyx* hemocytes.

In *Manduca*, cell division associated with the development of the eyes, leg primordia, and wing imaginal discs was suppressed by JH at the onset of the last larval stage (Truman *et al.*, 2006). The formation and growth of these primordia was also inhibited when larvae were starved, but was enhanced when the larvae began to feed. Koyama *et al.* (2004) reported that the cell cycle in wing discs of *Bombyx* was controlled by 20E. Our results show that hemocyte division is dependent on the larval developmental stage, suggesting that the *Bombyx* hemocyte cell cycle may also be under hormonal control.

Shifts in the hemocyte cell cycle

The DNA content of the hemocytes increased gradually toward the molting phase, and 8C hemocytes were observed. This suggests that endoreplication occurs in *Bombyx* hemocytes. By immunochemistry, I detected mitosis in circulating hemocytes. Nittono (1960) observed mitosis in prohemocytes, granulocytes, and, rarely, in spherulocytes. Most of the circulating hemocytes in the larvae were granulocytes (Fig. 3-5B), suggesting that the shift in DNA content of the hemocytes was largely due to that of the granulocytes. The percentage of 8C granulocytes decreased rapidly once the larvae entered the molting phase (Fig. 3-5C). The reason for this is probably that the mature 8C granulocytes finished their life span and were degraded by apoptosis or autophagocytosis (Okazaki *et al.*, 2006). Many lysosomes were detected in granulocytes at the molting phase using a lysosome-specific probe (data not shown), and several granulocytes with extremely numerous granules and active adhesions at the molting phase (Fig. 3-5A) were subsequently degraded. The proportion of those granulocytes was decreased in the newly ecdysed larvae. These granulocytes might be at the end of their life cycle. Dynamic changes in the DNA content of the granulocytes were observed only when the larvae entered the feeding and molting phases, which probably reflects the beginning and ending of DNA synthesis and cell division.

I was unable to document the prohemocyte cell cycle because so few prohemocytes were present in the hemolymph (Fig. 3-5B). Prohemocytes are predominantly located in the hematopoietic organ,

and they are believed to differentiate into granulocytes and plasmatocytes after entering the hemolymph (Yamashita and Iwabuchi, 2001; Ling *et al.*, 2005). The number of hemocytes in the hematopoietic organ and the volume of the organ increase gradually throughout the feeding period (Akai and Sato, 1971; Nakahara *et al.*, 2003), indicating that prohemocytes actively proliferate in the hematopoietic organ.

Those plasmatocytes with a 2C DNA content gradually shifted to become 4C and 8C (Fig. 3-5D). Few mitotic plasmatocytes were observed in hemolymph in previous studies and in my experiments; however, Gardiner and Strand (2000) reported that all types of hemocytes with the exception of oenocytoids synthesized DNA in larval *Pseudoplusia includens* and *Spodoptera frugiperda*. These data suggest that the increase in DNA content of the plasmatocytes is the result of DNA synthesis without mitosis. The drop in the number of mature 8C plasmatocytes at IV4 suggests that hemocyte renewal occurs during molting, as in the case of granulocytes.

Cell cycle arrest of hemocytes at a high temperature

Few mitotic hemocytes were observed and proliferation was inhibited at a high temperature (Figs. 3-1C and 3-3). The drop in proliferation was not much related to a nutritional deficiency because the larvae at 38°C continued to feed and increased their body weight (Fig. 3-1A). In addition, I observed no increase of DNA content in the hemocytes when larvae starved (Fig. S-13), whereas hemocytes in

the larvae reared at 38°C continued to synthesize DNA. These results suggest that the nutrition is sufficient for proceeding cell cycle. The rapid decrease in hemocyte growth following a shift in temperature from 26 to 38°C in the middle of the stadium also sustain the possibility that cell cycle was stopped by high temperature (Fig. 3-2B). More experiments, however, would be needed for denying effects of nutrition at high temperature.

The gradual accumulation of cells with a high DNA content occurred in the hemocytes of larvae reared at 38°C (Fig. 3-4). This indicates that at a high temperature, hemocytes carry out DNA synthesis but not mitosis. I previously found that division in BmN cells, which are derived from *Bombyx mori*, was arrested at a high temperature and that the arrest occurred during G_2 (CHAPTER 1). Hemocyte proliferation reverted to the normal state following a reduction in temperature from 38 to 26°C. This reflects the situation in BmN cells (cell cycle progression was recovered by a temperature shift from 38 to 26°C). Furthermore, high phosphorylation level of Cdc2 was observed in both hemocytes and BmN cells (Fig. 3-6). These *in vivo* and *in vitro* data indicate that a heat-induced cell cycle arrest occurs in *Bombyx* cells, resulting in developmental inhibition.

In Lepidoptera and other insects, fluctuations in hemocyte number are influenced by the release of hemocytes from the hematopoietic organ and attachment of the cells to tissues (Tu *et al.*, 2002; Okazaki *et al.*, 2006). The number of hemocytes in circulation can change rapidly in response to stress, wounding, or infection (Ratcliffe *et al.*, 1985; Lackie, 1988). As high temperatures are a

source of stress for insects, it is possible that the number of hemocytes was directly altered by the change in temperature. In fact, a two- to three-fold increase in hemocyte density was observed after momentarily immersing a silkworm in water heated to 50-55°C (Nittono, 1960). However, I observed a decrease in hemocyte density following exposure of silkworm larvae to 38°C for 24 h. I also confirmed that short-term exposure to 38°C (for 2 h) did not induce a significant change in hemocyte density (data not shown). The decrease in hemocyte density at 38°C may be due to a cell cycle arrest accompanied by an increase in hemolymph volume caused by feeding instead of being due to attachment of the cells to tissues. Nakahara *et al.* (2003) suggested that hemocytes are supplied from the hematopoietic organ immediately before ecdysis and throughout the feeding period. The hemocytes in the hematopoietic organ may stop proliferating at 38°C, and the number of cells released from the organ may also decline. The distribution of hemocyte types changed dynamically when the larvae were reared at 38°C; the percentages of granulocytes and spherulocytes decreased, while the percentage of plasmatocytes increased (Fig. 3-5B). The higher turnover of granulocytes and spherulocytes may result in this distribution change.

FIGURES AND TABLES

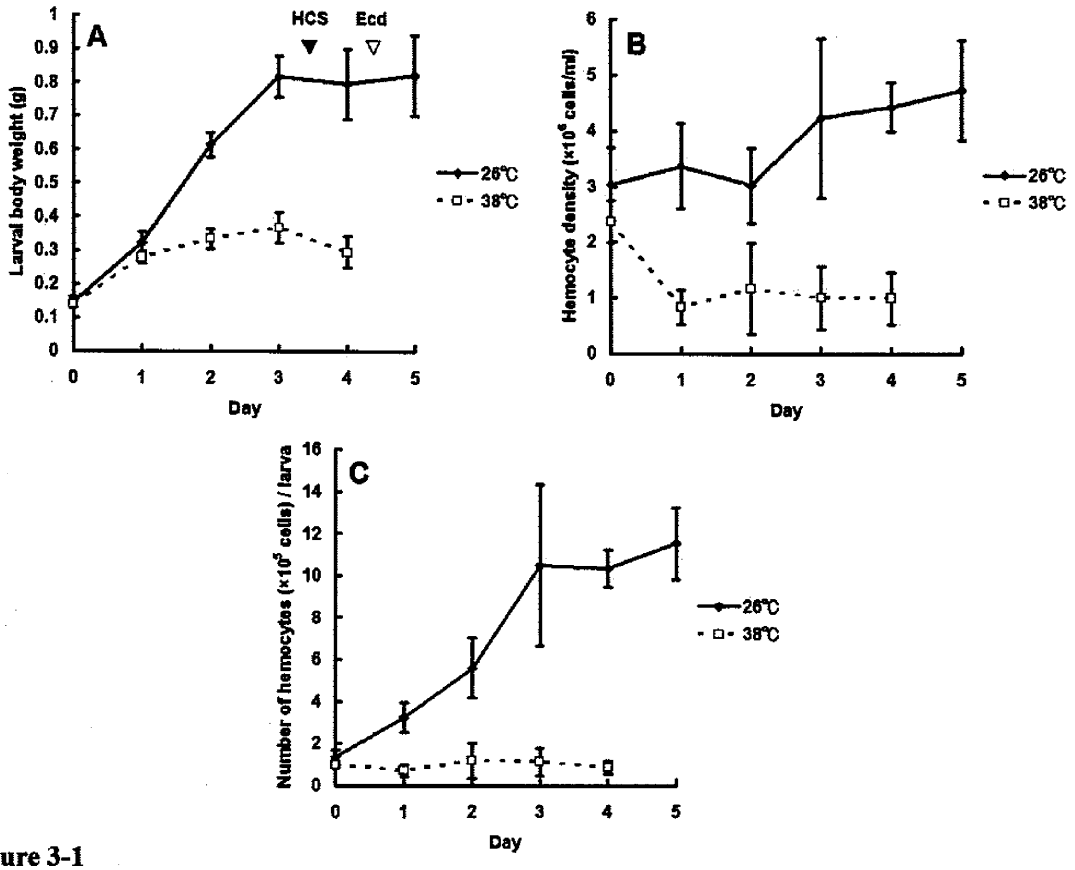

Figure 3-1

Effects of high temperature on the total number of hemocytes in fourth-instar silkworm larvae. (A) Larval body weight at 26 or 38°C (means ± SD, n = 6). The timing of head capsule slippage (HCS) and ecdysis (Ecd) are indicated by closed and open arrowheads, respectively. (B) Hemocyte density in the larval hemolymph at 26 or 38°C. (C) The total hemocyte number per larva estimated by multiplying the cell density in the hemolymph by the estimated hemolymph volume (approximately 30% of the larval body weight).

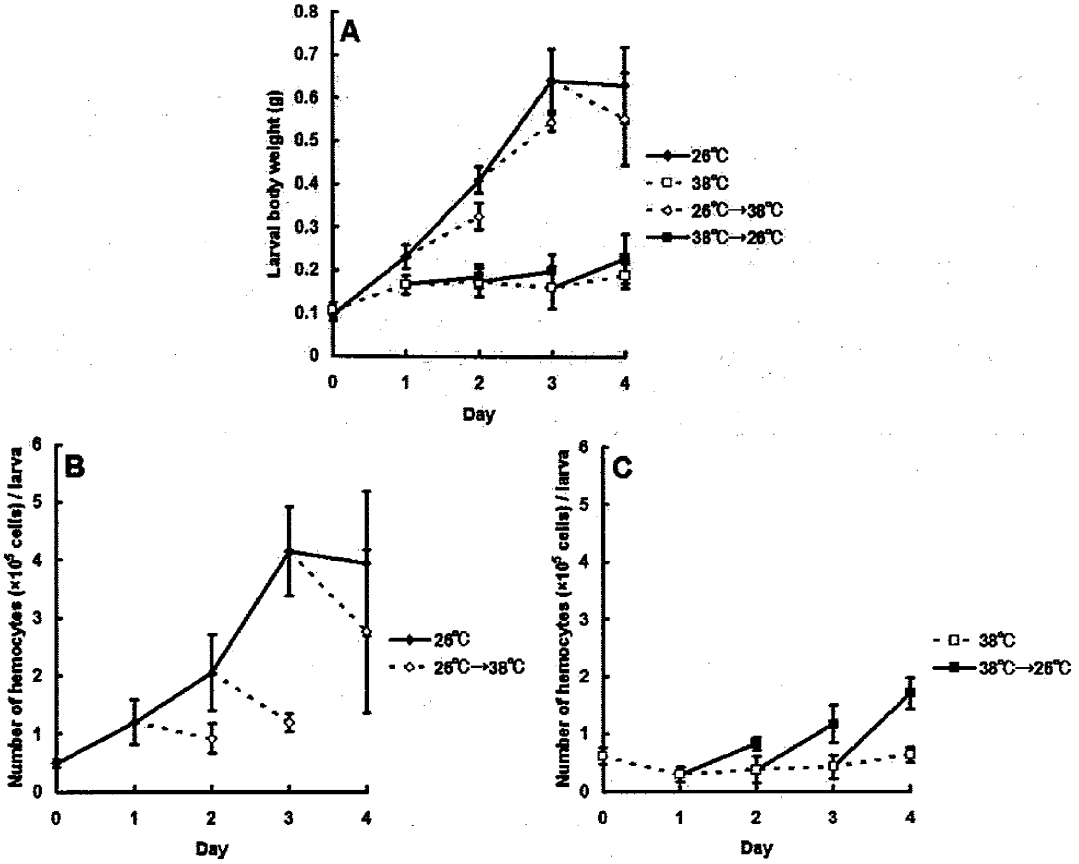

Figure 3-2

Effect of a temperature shift on body weight and the number of hemocytes in fourth-instar silkworm larvae. (A) Change in larval weight following the temperature shift (means ± SD, n = 6). (B, C) Change in the total hemocyte number following the temperature shift. The number of hemocytes was estimated from the larval weight and hemocyte density.

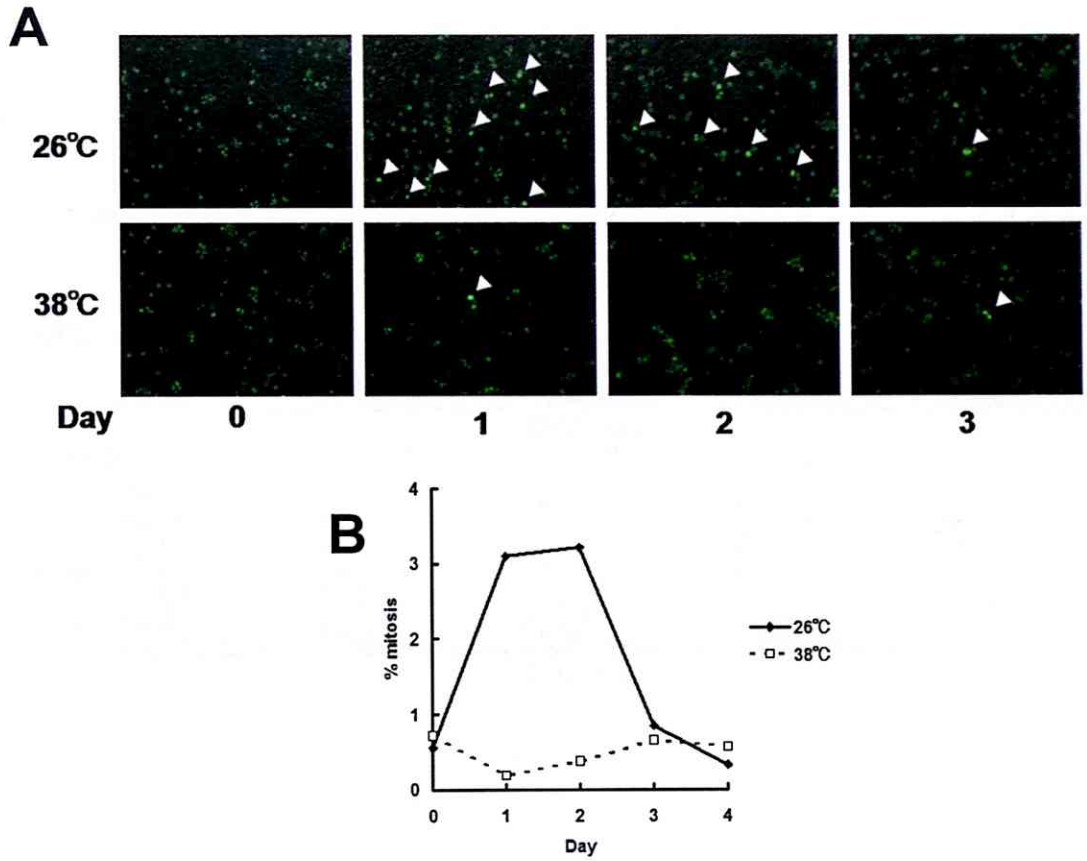

Figure 3-3

Detection of circulating mitotic hemocytes. (A) Silkworm larvae in the fourth instar were reared at 26 (upper row) or 38°C (lower row). Hemocytes were collected each day and immunostained with an anti-phospho-histone-H3 (Ser10) antibody. The green signals in immunostaining indicate individual cells and bright green signals with arrowheads indicate cells in M phase. (B) The proportion of mitotic hemocytes (n = 500).

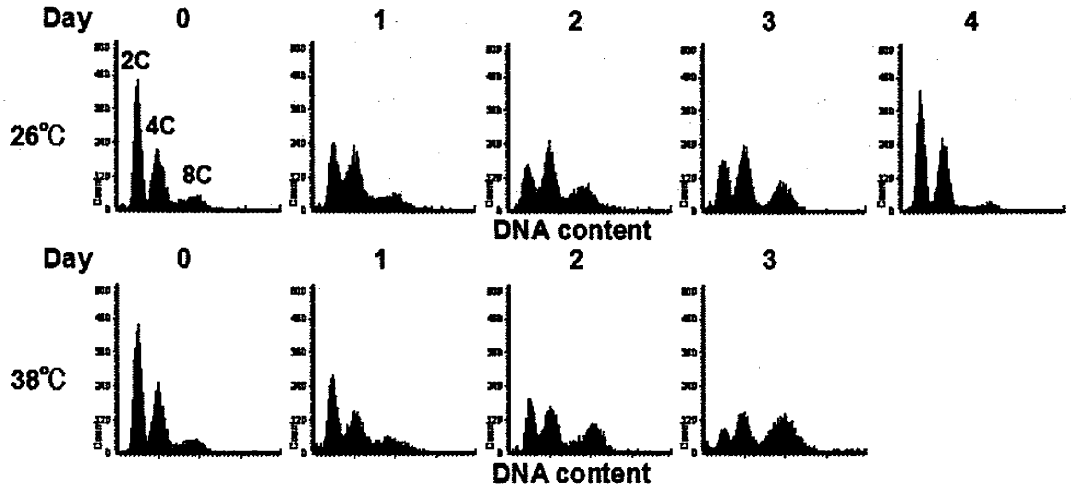

Figure 3-4

Changes in hemocyte DNA content in the fourth instar. Hemocytes were isolated from fourth-instar silkworm larvae (three males and three females) reared at 26 (upper row) or 38°C (lower row). The DNA content of approximately 10,000 hemocytes was analyzed by LSC; histograms were produced using LSC software. Abscissa, DNA content; ordinate, cell counts. The DNA content indicated above each peak is based on that of a spermatogonium (1C).

	26℃					38℃			
Day	0	1	2	3	4	0	1	2	3
2C (%)	45.2±4.3	34.3±3.1	31.9±4.4	31.3±2.8	42.8±4.6	48.4±1.3	45.5±5.7	29.5±0.9	17.7±3.5
4C (%)	38.1±0.5	44.8±1.4	43.4±2.1	42.6±1.9	42.8±3.8	38.2±0.8	34.5±2.1	35.6±2.4	36.0±0.9
8C (%)	16.7±3.9	20.9±2.6	24.7±2.4	26.1±2.8	14.4±5.9	13.4±2.1	20.0±3.7	34.9±1.9	46.3±4.4

The values listed are the means ± SD (n = 3).

Table 3-1

The percentages of hemocytes with a 2C-8C DNA content in fourth-instar silkworms.

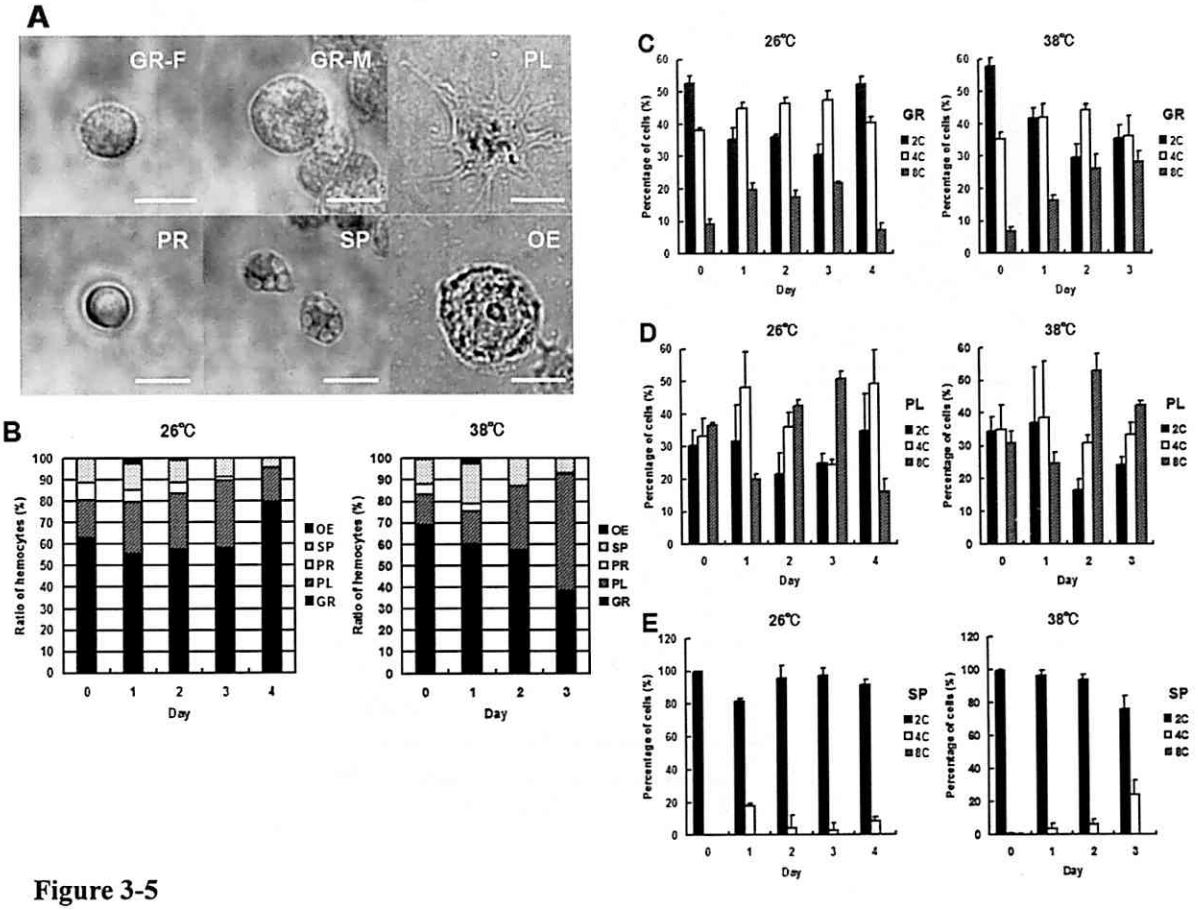

Figure 3-5

Changes in hemocyte type in the fourth instar. (A) Representative images of the five types of hemocytes found circulating in fourth-instar silkworm larvae. GR-F, granulocyte in the feeding phase; GR-M, granulocyte in the molting phase; PL, plasmatocyte; PR, prohemocyte; SP, spherulocyte; OE: oenocytoid. Scale bar = 10 μm. (B) Hemocyte composition in the fourth larval stage at 26 or 38°C. The hemocytes were counted after LSC (Fig. 3-4) (n = 300). DNA content in the granulocytes (C), plasmatocytes (D), and spherulocytes (E) (means ± SD, n = 3). The types of hemocytes with each DNA content from Figure 3-4 were classified.

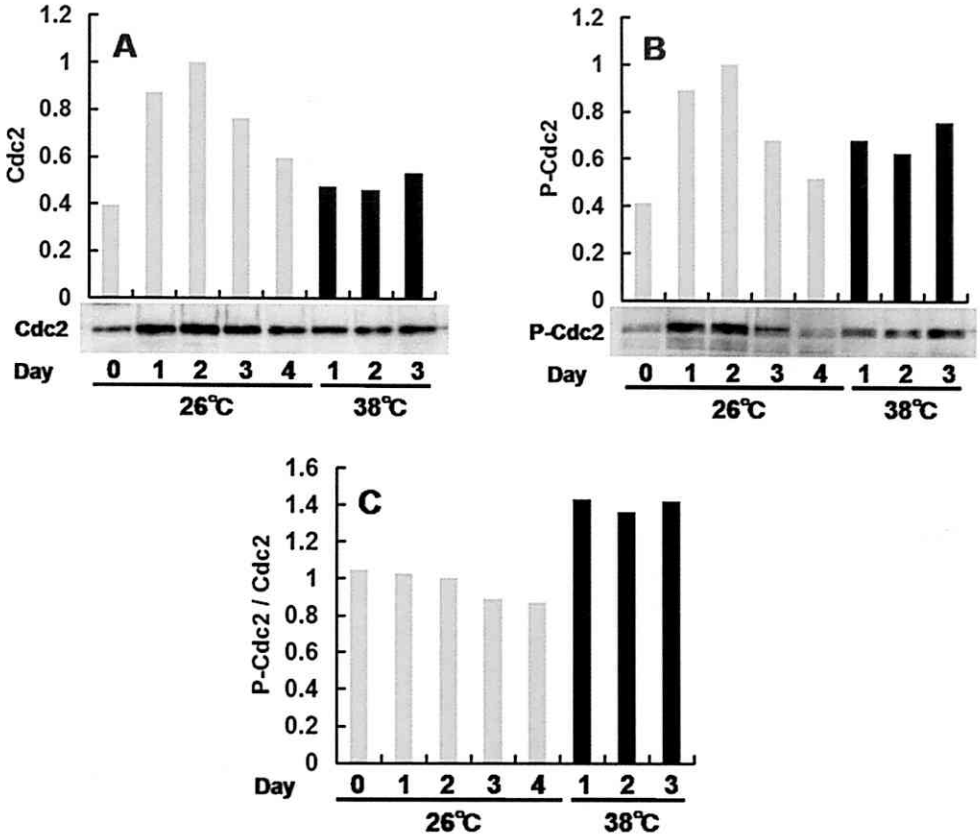

Figure 3-6

Effect of temperature on phosphorylation level of Cdc2 in the silkworm hemocyte. Hemocytes were isolated from fourth-instar larvae reared at 26 or 38°C. The hemocytes were then dissolved in sample buffer and analyzed by Western blotting using antibodies against (A) total-Cdc2 (PSTAIRE) or (B) phospho-Tyr15-Cdc2. The blot presented is representative of three separate experiments. Blots were analyzed by Scion Images. The relative densitometric readings (IV2 = 1) are presented in the upper panel. (C) Phosphorylation level of Cdc2 was represented by dividing the densitometric reading of phospho-Cdc2 into the total-Cdc2.

GENERAL DISCUSSION

I focused on high temperature stress on insect cell cycle. Temperature, however, has many effects on insect development. In this chapter, I present other results concerning temperature effects on the silkworm physiology and discuss about several aspects of heat effects on insects.

Effects of high temperature on endocrine and nervous system

In this study, effects of temperature on endocrine and nervous system were excluded by using of insect cell line and the direct effects on insect cells were observed. However, effects on these systems are not negligible in discussion of the cell division in the silkworms.

When larvae at day 0 of the fourth larval stage were reared at 34-36°C, the larvae failed to enter the molting phase and continued to feed and grow beyond the weight limit of the fourth larval stadium (Figs. 1-1 and S-12). Furthermore, newly ecdysed larvae in the fourth-instar which were reared for 48 h at 38°C and then maintained at 26°C also lost the fourth molting and almost all the larvae changed to tri-molter (a silkworm molting three times during a larval period before metamorphosis; Figs. S-1, S-2 and S-3). No tri-molter emerged when larvae in the first, second, or third instar are reared for 48 h at 38°C (Table S-1). Moreover, when the heat-treated larvae, which showed prolonged fourth-instar larval period, were injected with 20-hydroxyecdysone, the larvae

immediately entered the molting phase and showed head capsule slippage (Fig. S-4). These suggest that the secretion of ecdysteroid from prothoracic gland is stopped by heat exposure only at the fourth instar. Tri-molter adults had smaller body size (Fig. S-2) but shorter larval period than tetra-molter. The stop of ecdysteroid secretion by high temperature accelerates the growth to adult and the reproductive behavior. Normal mating and oviposition were observed in tri-molter and there was no serious trouble in next life cycle.

Thermal receptor of insects is present on antennae and probably whole-body vibrissae (Chapman 1998). Many transient receptor potential (TRP) ion channel family proteins which have variable numbers of ankyrin repeats in the N terminal and exhibit a putative 6-transmembrane structure are identified through the animal kingdom (Littleton and Ganetzky, 2000; Montell, 2003). It is proposed that TRP family proteins function as crucial sensors of temperature. I screened *Bombyx* whole-genome database for the homologous proteins to *Drosophila* TRP family proteins and identified some genes. RT-PCR cleared that these genes expressed strongly in head and the antennae (data not shown). Although more precise experiments are needed, it is possible that the sense of high temperature on antennae or brain suppressed ecdysteroid secretion or synthesis. *In vitro* cultures of prothoracic gland and transplant experiments of brain would reveal the mechanisms.

In this way, it is obvious that endocrine and nervous system are affected by temperature. Cell division in insects is controlled by ecdysteroid and juvenile hormone (Koyama *et al.* 2004; Truman

et al. 2006). It is possible that high temperature changes hormonal regulation in silkworm larvae and then inhibits cell division of the hemocytes. However, arrest of cell cycle in G_2 phase and high phosphorylation level of Cdc2 were observed in hemocytes of heat-treated silkworm (CHAPTER 3), in agreement with the effects of high temperature on the cell line (CHAPTER 1 and CHAPTER 2). These results suggest that heat-activated G_2/M checkpoint pathway is most significant causes for arrest of cell division in hemocytes.

Effects of high temperature on enzyme activity

This study proposes that the heat-induced cell cycle arrest should be one of the crucial causes for the growth arrest of insects under high temperature stress. However, other troubles caused by high temperature might inhibit silkworm growth together with cell cycle arrest. Enzyme activity of living organism is greatly influenced by temperature (Schmidt-Nielsen, 1997). Denaturation of enzymes in the silkworm is most likely to prevent the growth. From this reason, stabilities of activities against temperature were examined in three *Bombyx* enzymes, amylase, phosphatase, and trehalase (Fig. S-5). Phosphatase and trehalase activity increased with temperature but amylase activity had the highest point at 26-30°C, namely optical temperature of amylase was correlated with adequate temperature for silkworm growth. These results suggest that two types of enzyme are existence in insects: Type 1 is an enzyme whose activity increased with temperature and is not related to the

insect activity. Type 2 is an enzyme whose activity is correlated with growth temperature of insects. Because of a few examined enzymes, it is not clear which type of enzyme is the majority in insects. However, the existence of type 2 may induce growth arrest of insects. Both types of enzyme retained their activity for at least 1 h at 38°C (Fig. S-6) but the total activity decreased in silkworms reared at high temperature (Fig. S-7). Enzyme activity also decreased in starved silkworms at high temperature (Fig. S-8). Since enzymes are markedly consumed under high temperature condition, silkworms might be deficient in enzymes, become frailty, and finally die. The growth arrest of heat-treated silkworms is possibly caused by various factors involved in cell cycle arrest, enzyme inactivity, and so on.

Cell cycle arrest in insect cell caused by various stresses

Cell cycle arrest in G_2 phase was also observed when BmN cells cultured at low temperatures (CHAPTER 1). Moreover, low temperature induced phosphorylation of p38 and accumulation of phosphorylated Cdc2 (Fig. S-10). Although the effects are weaker than those of high temperature, these results show that the same mechanisms as heat-treated cell cycle arrest are activated by low temperature stress. Lee *et al.* (2005) showed that silkworm thioredoxin peroxidase, which was an antioxidant enzyme, was induced by low or high temperature stimulus. Detection of reactive oxygen species (ROS) using DCFH-DA would disclose whether oxidative stress causes cell cycle arrest at

low temperature.

Continuation of insect cell culture to confluent also induced cell cycle arrest in G_2 phase (data not shown). The strong fluorescence was detected in condensed cells by treatment with DCFH-DA, resulting that ROS generated in those cells. Cell cycle arrest in confluent cultures as well as heat-exposure cultures is presumably caused by oxidative stress.

Infection of insect cell lines with *Autographa californica* multiple nucleopolyhedrovirus resulted in increased levels of lipid hydroperoxides and protein carbonyls which were the evidence of oxidative stress (Wang *et al.*, 2001). On the other hand, cell cycle arrest of BmN cells at G_2/M phase occurs in *Bombyx mori* nucleopolyhedrovirus (BmNPV) infection (Baluchamy and Gopinathan, 2005). Furthermore, Katsuma *et al.* (2007) showed phosphorylation of p38 was observed in BmN cells infected with BmNPV. In these points, there is close relationship between generation of oxidative stress and G_2 arrest in viral infection of insect cells.

Additionally, I found that deficiency of calcium induced G_2 arrest in BmN cells (Fig. S-9). Although relationship between calcium deficiency and oxidative stress are unknown, detection of ROS using DCFH-DA and p38 phosphorylation using Western Blotting would elucidate the mechanism.

Fujiwara *et al.* reported that temperature-dependent activation of ERK and p38 occurred in embryonic diapause initiation and termination in *Bombyx* (2006). Slow accumulation of

phosphorylated p38 was observed in BmN cells by low temperature stress at 10°C (Fig. S-10). In initiation or termination of diapause may be regulated by reactivity of MAPKs to temperature shift. Interestingly, most cells of diapause-destined embryos are arrested during the G_2 cell cycle stage (Nakagaki *et al.*, 1991). It is possible that same mechanism act in G_2 arrest caused by both high temperature stress and diapause.

Differences in tolerance to temperature among *Bombyx* strains

Many silkworm races and strains are present and maintained. A Chinese strain, Shoon, was used in this study and the hemocyte proliferation at high temperature was examined. The same experiment was also performed using a subtropical strain, N4, resulting that there was a little difference between these strains. At 36°C, larval body weights of both strains were increased (Fig. S-12A). On the other hand, the number of hemocytes was increased in N4 but suppressed in Shoon (Fig. S-12B). Additionally, N4 showed late head capsule slippage while Shoon continued to feeding without entering molting phase. These results suggest that cells in N4 are more resistant to high temperature stress than in Shoon. Synthesis of antioxidant enzyme occurs in silkworm at stress temperatures (Lee *et al.*, 2005), namely silkworms have resistant mechanisms to high temperature. Considering that high temperature induced oxidative stress and cell cycle arrest in *Bombyx* cells, it is speculated that N4 is more tolerant to heat-induced oxidative stress than Shoon. Interesting results

would be obtained to compare the expressions of antioxidant enzymes between both strains. Taking advantage of abundant strains, the properties of thermal tolerant strains are expected to be cleared.

Influence of cell cycle arrest on insect growth

In this study, the effects of temperature on proliferation of insect cell line were investigated to understand the effects of temperature on insect growth. Although use of BmN cells simplifies the temperature reactions in silkworms, there is a question whether the reactions reflect that of *in vivo* cells. Heat-induced cell cycle arrest was observed in silkworm hemocytes as well as BmN cells (CHAPTER 3). Cell cycle arrest of hemocyte is possibly caused by the same mechanism as BmN cells. The silkworm fed artificial diet containing cell cycle inhibitor, hydroxyurea, halted both hemocyte proliferation and the body weight gain (Fig. S-11). This result represents close relationship between cell cycle arrest and growth arrest of silkworms. However, increase in larval body weight was observed at impaired temperature for hemocyte proliferation (Fig. S-12). Since the expression levels of antioxidant enzymes differ in silkworm tissues (Lee *et al.*, 2005; Yamamoto *et al.*, 2005), the sensitivity to high temperature presumably differ in cell types. It is important to confirm the heat-induced arrest of cell division in various tissues using M phase marker, anti-phospho-histone-H3 (Ser10) antibody. This verification would provide evidence that the cell cycle arrest by high temperature is common phenomenon in silkworm cells and clear correlation between cell

division and silkworm growth.

SUPPLEMENTAL FIGURES AND TABLES

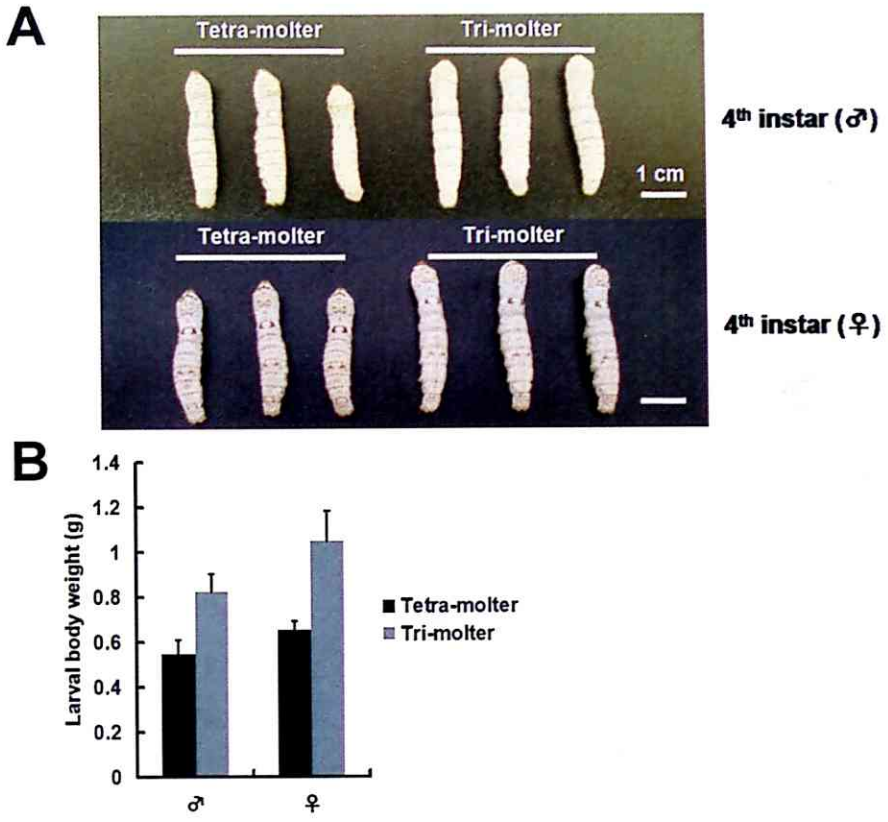

Figure S-1

Precocious metamorphosis in the silkworms by high temperature. *Bombyx mori* silkworm larvae Shoon were reared on an artificial diet under 12 h light and 12 h darkness (12L-12D) photoperiod at 25°C until the third instar. Twenty newly ecdysed larvae in the fourth instar were then transferred to a plastic case (20.5 cm × 15.0 cm × 5.2 cm) and reared at 26 or 38°C without light for 48 h. Then, the larvae were maintained at 26°C. Heat treated larvae continued feeding for 8 days and then metamorphosed to pupae (tri-molter: a silkworm molting three times during a larval period before metamorphosis), while control larvae entered the larval molting phase and showed head capsule slippage after 2 days (tetra-molter). (A) Photographs of larvae in the fourth molting phase (left) and

larvae just before precocious metamorphosis (right). Upper lane: male, lower lane: female. Scale bar = 1 cm. (B) Maximum body weight of the fourth larval stage (means ± SD, n = 16).

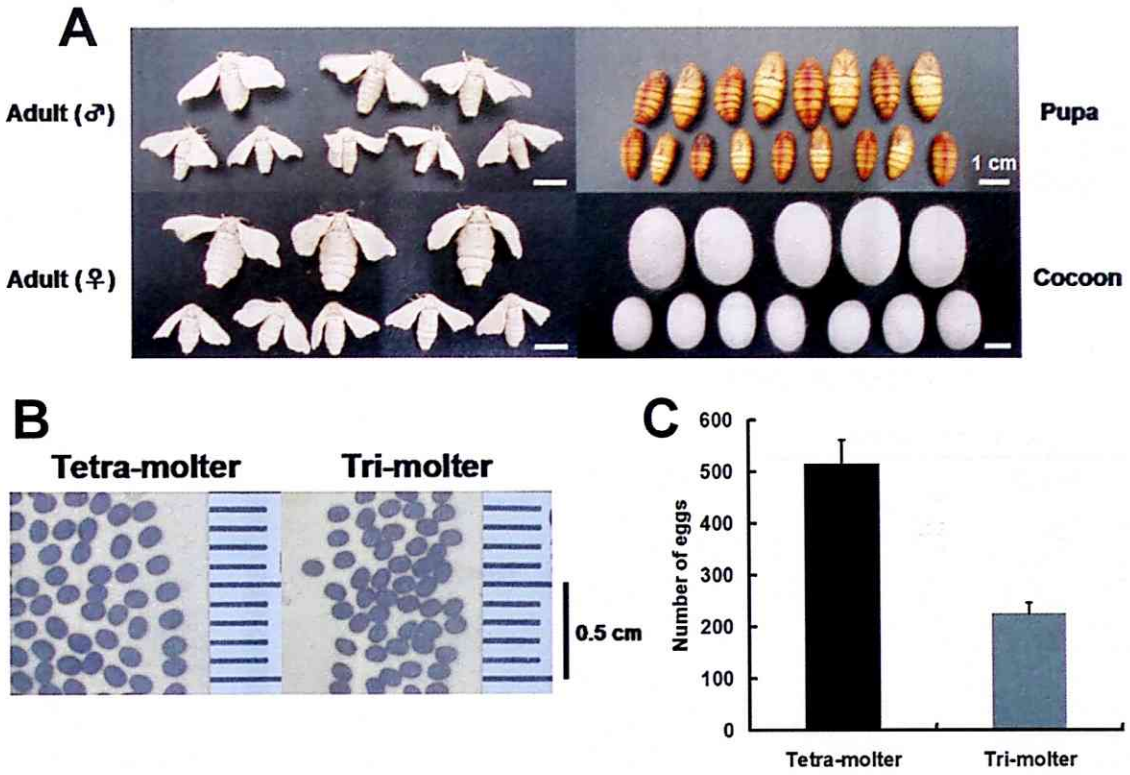

Figure S-2

Characteristics of tri-molter caused by high temperature. (A) Photographs of adult, pupa, and cocoon. Upper lane: tetra-molter, lower lane: tri-molter. Scale bar = 1 cm. (B) Sizes of eggs laid by tri-molter (right) or tetra-molter (left). Scale bar = 0.5 cm. (C) Number of eggs laid by tri-molter or tetra-molter (means ± SD, n = 9).

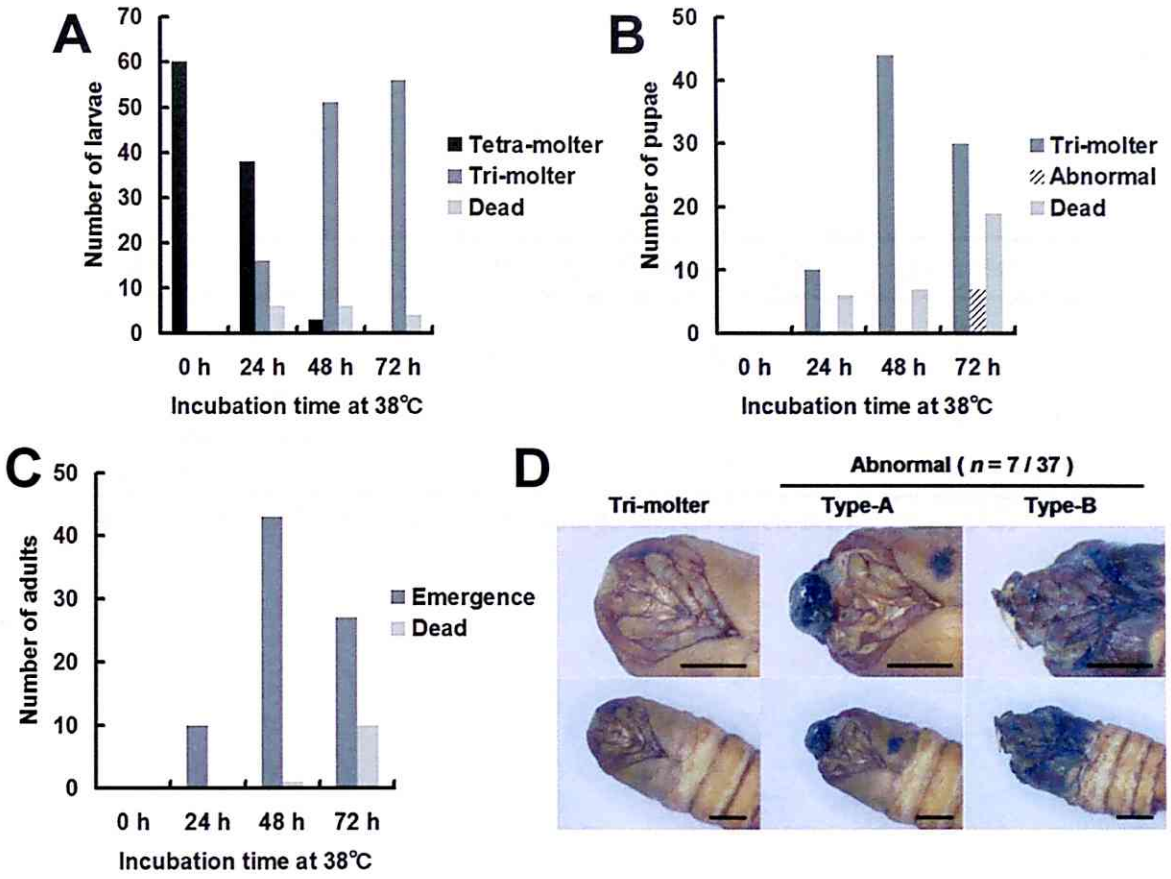

Figure S-3

Occurrence of tri-molter by high temperature. Twenty newly ecdysed larvae in the fourth instar were transferred to a plastic case (20.5 cm × 15.0 cm × 5.2 cm) and reared at 38°C without light for indicated times. After heat treatment, larvae were maintained at 26°C. Effect of heat exposure time on (A) occurrence of tri-molter, (B) tri-molter pupation, and (C) the emergence to adult. (D) Abnormal pupation of tri-molter by heat treatment for 72 h. Scale bar = 0.5 cm.

Stage	*n*	Tetra-molter (%)	Tri-molter (%)	Dead (%)
I	80	100	0	0
II	80	100	0	0
III	80	100	0	0
IV (♂)	40	7	89	4
IV (♀)	40	3	92	5

Larvae at day 0 of the each larval stage were reared for 48 h at 38°C.

Table S-1

Effect of heat treatment on each stage of larvae. Larvae at day 0 of the each larval stage were reared at 38°C for 48 h without light and then transferred to 26°C.

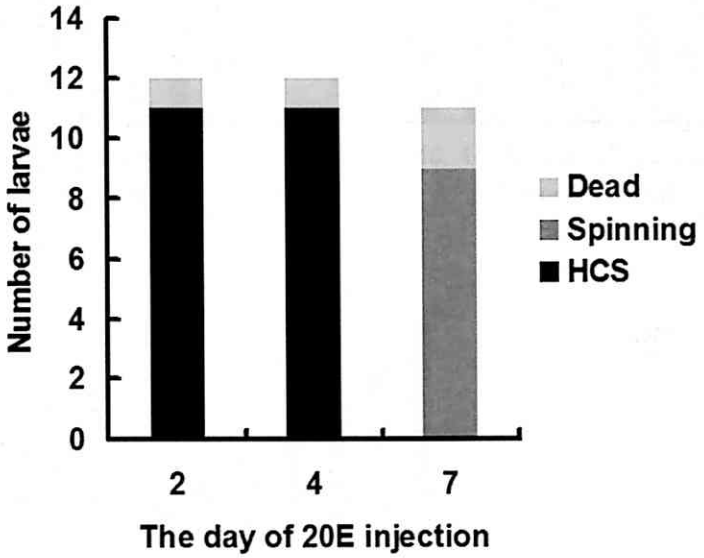

Figure S-4

Effect of molting hormone on heat-induced tri-molter larvae. Newly ecdysed larvae in the fourth instar were reared at 38°C for 48 h. These heat-treated larvae were mostly destined to undergo metamorphosis without the fourth molting (Table S-1). After transferred to 26°C, the larvae were injected with 20-hydroxyecdysone (20E) at the indicated time. Head capsule slippage (HCS) is a signal of the beginning of larval molting process. Spinning is a signal of the beginning of pupation.

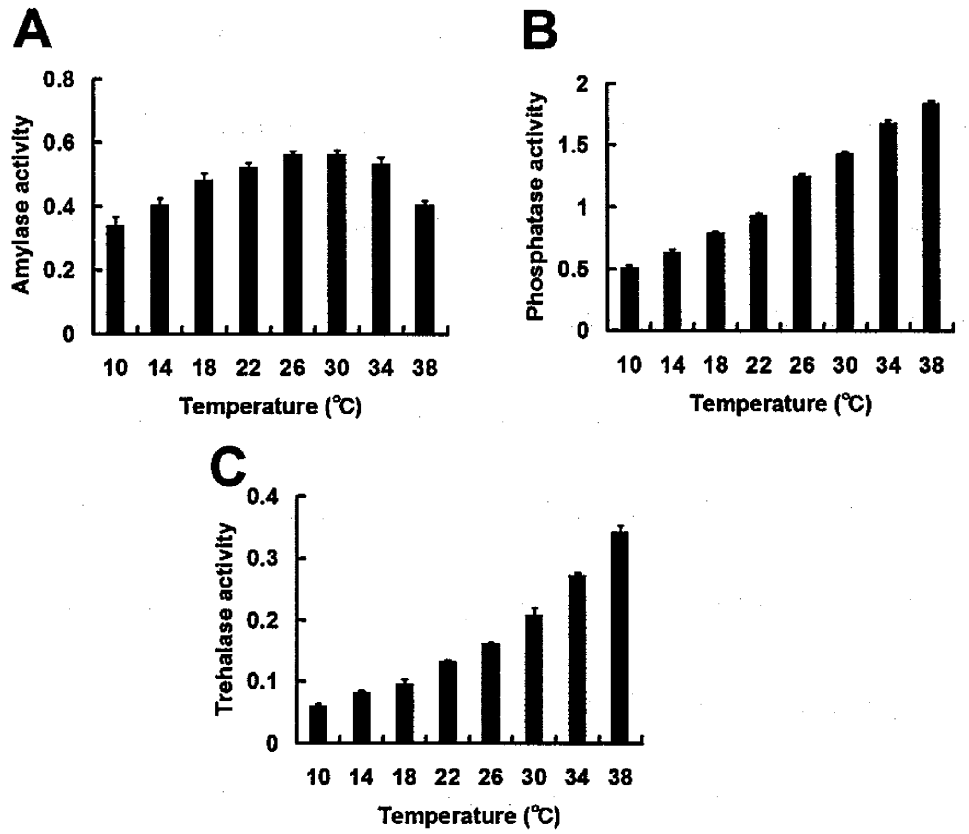

Figure S-5

Optimal temperature for several enzyme activities in the silkworm. Silkworms were reared on an artificial diet under a 12L-12D at 25°C until day 1 of the fifth larval instar. The hemolymph (five males and five females) was collected from incisions made in the abdominal legs and centrifuged at 4.000 rpm for 10 min at 4°C to remove hemocytes and debris. The supernatant was used as enzyme solution for amylase and phosphatase. For analysis of trehalase activity, midguts were dissected (five males and five females), washed with cold DDW, and collected in a 1.5 ml tube on ice. The midguts were added with an equal volume of DDW, homogenized on ice and centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant diluted two-fold with DDW was used as enzyme solution. (A) Amylase

activity in hemolymph was measured by using an amylase assay kit obeyed Iodo-Starch method (Wako Pure Chemical Industries). Five μl of enzyme solution was added to 100 μl substrate solution preincubated for 10 min at various measurement temperatures and then mixed well. The mixture was incubated for 15 min at various temperatures in a water bath and then added with color development solution. The reacted solution was placed immediately into a well of a 96-well microplate. The absorbance at 655 nm was measured using a Microplate Imaging System (Bio-Rad, Laboratories). Amylase activity is calculated by the Caraway method (Caraway, 1959; means ± SD, n = 3). (B) Phosphatase activity in hemolymph was measured by the following method. Twenty μl of 0.05 M p-nitrophenylphosphate disodium salt solution was mixed with 40 μl of acetate-sodium acetate buffer solution (pH 4.7) and the substrate mixture was preincubated at measurement temperatures for 10 min. After the substrate solution was preincubated for 10 min, 5 μl of enzyme solution was added to the solution and mixed well. The mixture was incubated for 15 min at various temperatures in a water bath and then added with 1 ml of 0.1 M Na_2CO_3 solution to stop the enzyme reaction. The reacted solution was placed immediately into a well of a 96-well microplate, and then the absorbance at 415 nm was measured. Phosphatase activity is represented by the absorbance at 415 nm which shows p-nitrophenol amounts produced by phosphatase (means ± SD, n = 3). (C) Trehalase activity in midguts was measured by the following method. Twenty μl of acetate-sodium acetate buffer solution (pH5.6) was mixed with equal volume of 0.05 M trehalose solution. After the substrate

solution was preincubated for 10 min, 20 μl of enzyme solution was added to the solution and mixed well. The mixture was incubated for 15 min at various temperatures and then boiled for 5 min to stop the reaction. The reaction mixture was ice-chilled and centrifuged at 15,000 rpm for 5 min at 4°C. The amount of glucose produced by trehalase was measured by using Glucose C2 kit obeyed Mutarotase-GOD method (Wako Pure Chemical Industries). The 10 μl of supernatant was added to the 500 μl of color reagent preincubated for 10 min at 37°C and mixed. After incubation for 5 min at 37°C, the reacted mixture was placed immediately into a well of a 96-well microplate and absorbance at 490 nm was measured. Trehalase activity is represented by the absorbance at 490 nm which shows glucose amounts produced by trehalase (means ± SD, n = 3).

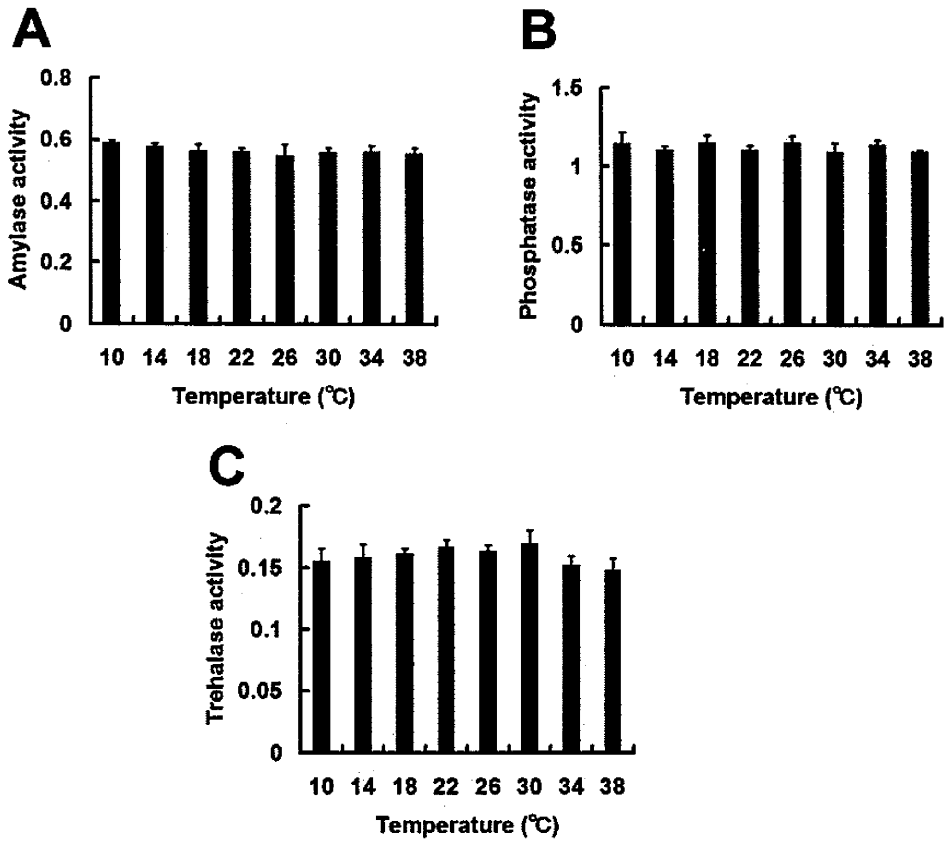

Figure S-6

Stability of silkworm enzymes against temperature. Enzyme solution was incubated for 1 h at various temperatures and then enzyme activities were measured. Each enzyme assay was followed by the methods described in figure S-5. All enzyme reactions were performed at 26°C. (A) Amylase activity, (B) Phosphatase activity, and (C) Trehalase activity (means ± SD, n = 3).

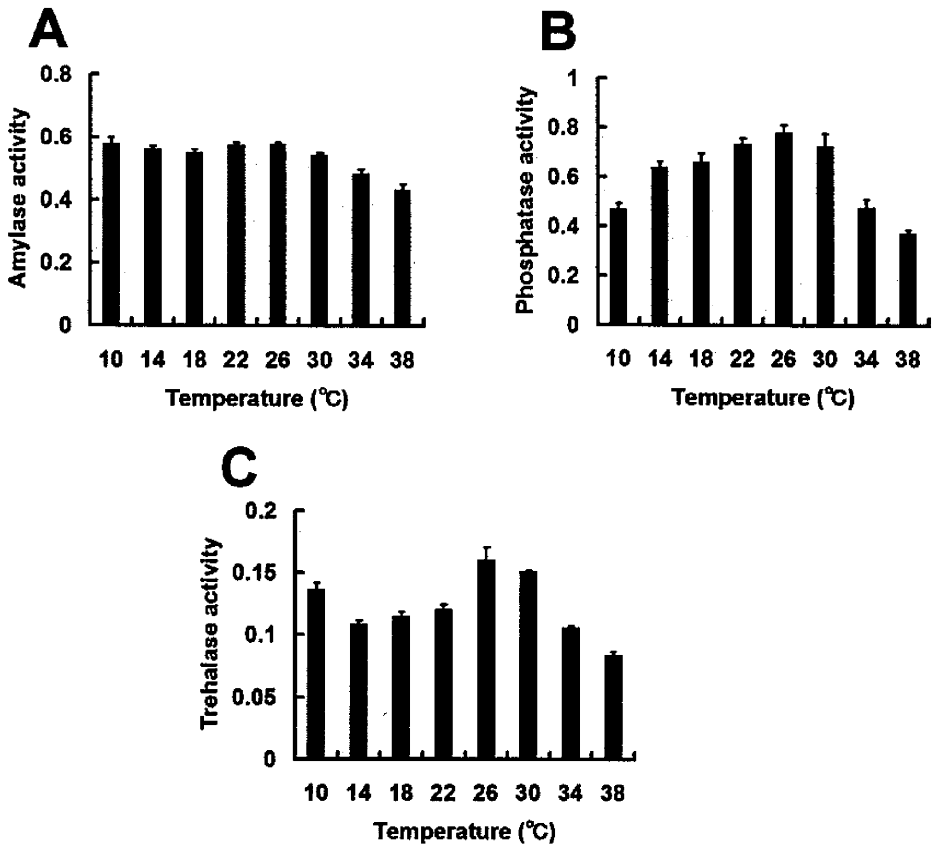

Figure S-7

Enzyme activities in silkworm larvae reared at various temperatures. Silkworms at day 1 of the final larval stage were reared on an artificial diet for 24 h at indicated temperatures without light and then enzyme solutions were prepared (three males and three females). Each enzyme assay was followed by the methods described in figure S-5. All enzyme reactions were performed at 26°C. (A) Amylase activity, (B) Phosphatase activity, and (C) Trehalase activity (means ± SD, n = 3).

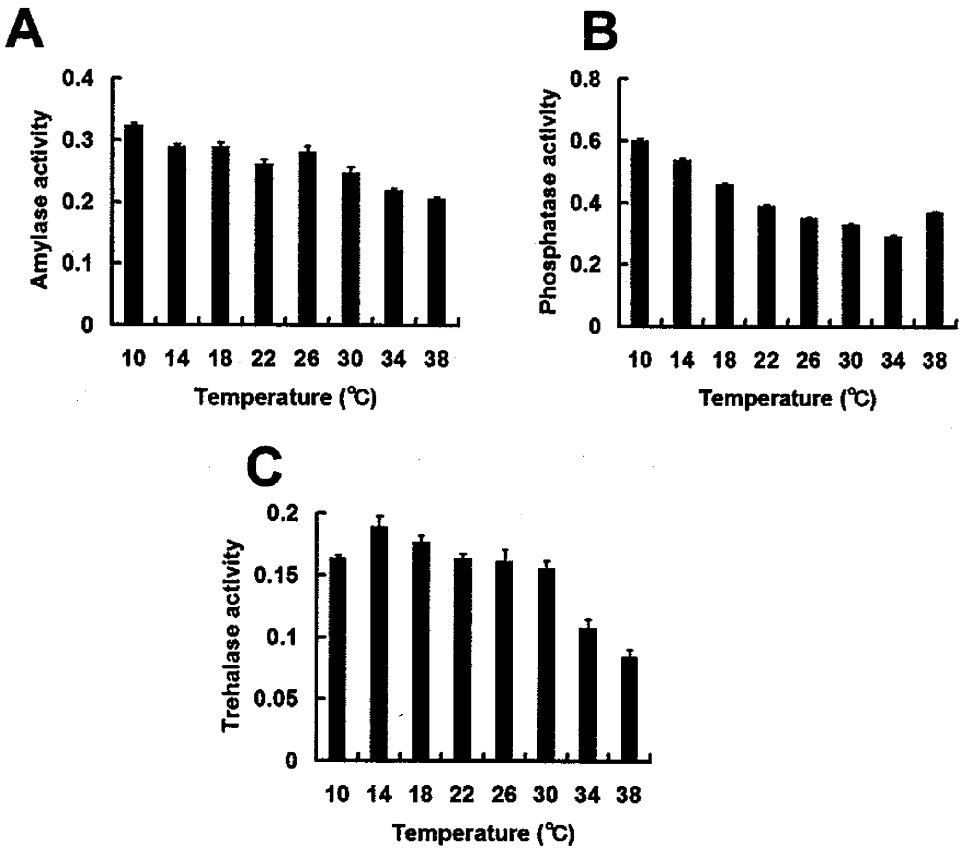

Figure S-8

Enzyme activities in silkworm larvae starved at various temperatures. Silkworms at day 1 of the final larval stage were incubated without artificial diet for 24 h at indicated temperatures and then enzyme solutions were prepared (three males and three females). Each enzyme assay was followed by the methods described in figure S-5. All enzyme reactions were performed at 26°C. (A) Amylase activity, (B) Phosphatase activity, and (C) Trehalase activity (means ± SD, n = 3).

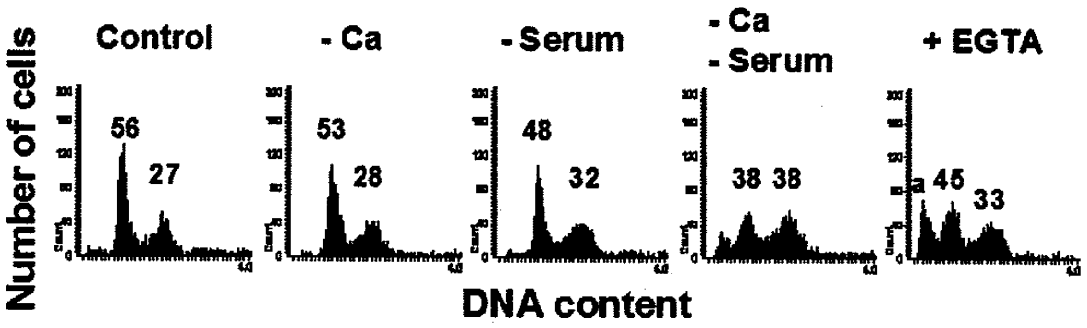

Figure S-9

Calcium deprivation effect on cell cycle of BmN cells. BmN cells were maintained in IPL-41 medium (J R Scientific, Inc., Woodland, CA, USA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic at 25°C. The medium was exchanged to IPL-41 medium without $CaCl_2$ (-Ca), FBS (-Serum) or both (-Ca, -Serum). For calcium chelation, o, o'-bis(2-aminoethyl) ethyleneglycol-N, N, N', N'-tetraacetic acid (EGTA; Wako Pure Chemical Industries) was added to the medium to a final concentration at 10 mM (+EGTA). After incubation for 72 h at 26°C, cell cycle analysis was performed (see CHAPTER 1, MATERIALS AND METHODS). The percentages of cell cycle in G_1 and G_2/M phase are indicated above each peak (a: apoptotic cells or cell debris).

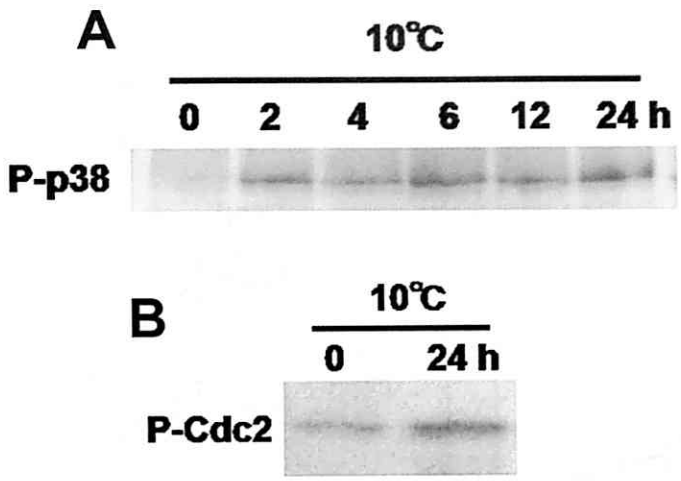

Figure S-10

The effect of low temperature on p38 and Cdc2 phosphorylation. BmN cells were cultured for indicated time at 10°C. The cells were then dissolved in sample buffer and analyzed by Western blotting using antibody against (A) phospho-p38 or (B) phospho-Tyr15-Cdc2 (see CHAPTER 2, MATERIALS AND METHODS).

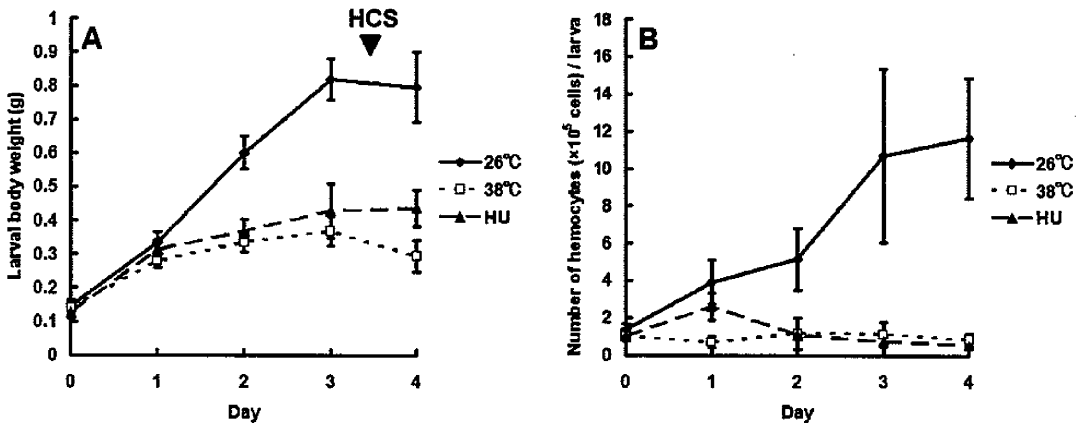

Figure S-11

Effect of high temperature and hydroxyurea on hemocyte proliferation. (A) Newly ecdysed silkworm larvae in the fourth instar were reared on an artificial diet at 26 or 38°C without light. Hydroxyurea was supplied by dropping the 1 mM solution on an artificial diet. Fresh food was fed each day to avoid deterioration of hydroxyurea and the larvae were reared at 26°C. The weight of each larva (three males and three females) was measured every day (means ± SD). The timing of head capsule slippage (HCS) is indicated by closed arrowhead. No larvae fed with the hydroxyurea-mixed diet or reared at 38°C entered the molting phase for 4 days. (B) The total hemocyte number per larva estimated by multiplying the cell density in the hemolymph by the

estimated hemolymph volume (approximately 30% of the larval body weight; see CHAPTER 3, MATERIALS AND METHODS).

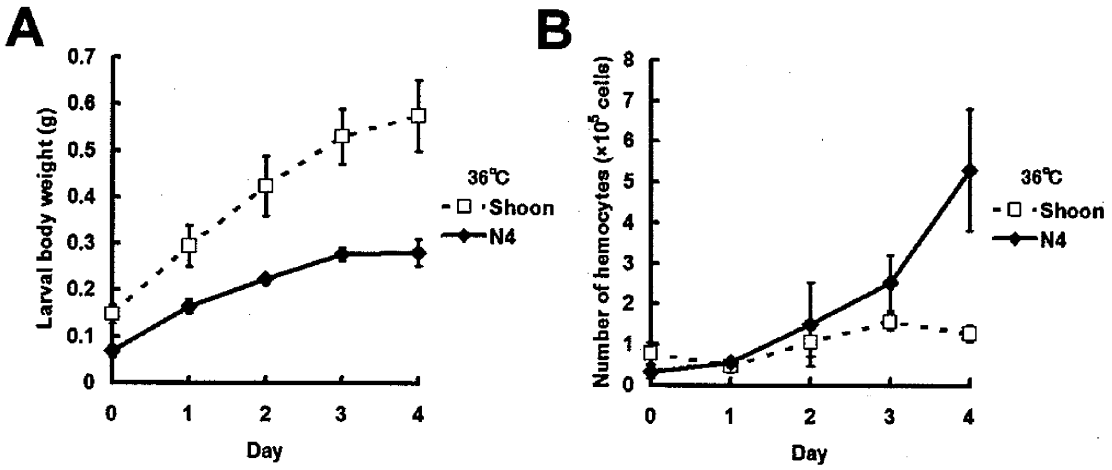

Figure S-12

Comparison of hemocyte proliferation between *Bombyx* strains. (A) Shoon and N4 were reared on an artificial diet until the third instar. Twenty newly ecdysed larvae in the fourth instar were then transferred to a plastic case (20.5 cm × 15.0 cm × 5.2 cm) and reared at 36°C without light and individual larval weights were measured (means ± SD, n = 6). Head capsule slippage was not observed in both of strains for 4 days. Although N4 shows late head capsule slippage at day 5, they failed to ecdyse and died with body color changing from yellow to orange. (B) The total hemocyte number per larva estimated by multiplying the cell density in the hemolymph by the estimated hemolymph volume (approximately 30% of the larval body weight; see CHAPTER 3, MATERIALS AND METHODS).

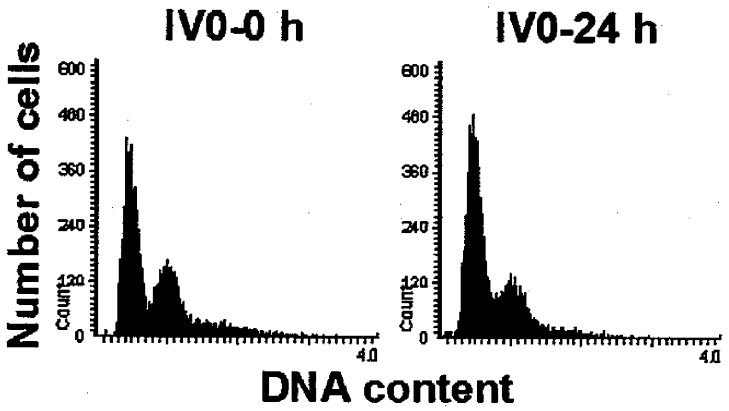

Figure S-13

Changes in hemocyte DNA content in the starved larvae. Hemocytes were isolated from newly ecdysed larvae in the fourth instar (IV0-0 h) or the larvae starved for 24 h at 26°C (IV0-24 h). The DNA content of approximately 10,000 hemocytes was analyzed by LSC (see CHAPTER 3, MATERIALS AND METHODS).

CONCLUSIONS

In this study, effects of high temperature stress on insect cell were investigated. Using insect cultured cell system, the cell cycle arrest in G_2 phase caused by high temperature was observed. The arrest mechanism is indicated on Figure C-1. High temperature stress induced reactive oxygen species generation in BmN cells. The oxidative stress caused damage to protein, lipid membranes, and especially DNA. These oxidative stresses activated G_2/M checkpoint pathway mediated by p38 MAPK. Phosphorylated p38 probably inactivated Cdc25 phosphatase, resulting that phosphorylated Cdc2 accumulated in BmN cells. The accumulation of inactive Cdc2 inhibited transition from G_2 to M phase. The same or a similar mechanism probably occurred in hemocytes of *Bombyx* larvae reared under heat stress condition. I conclude that the cell cycle arrest in G_2 phase by high temperature stress is a main reason for growth arrest of insects under high temperature condition.

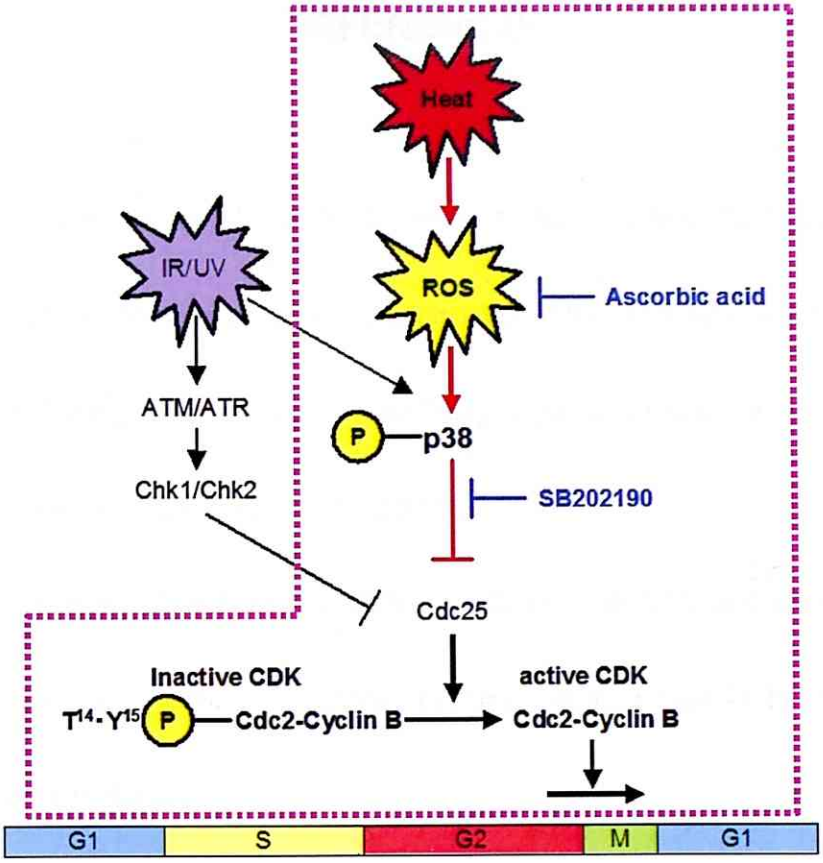

Figure C-1

Checkpoint pathway activated by high temperature stress in insect cells. The pathway concluded in this study is enclosed by dotted line. ROS: reactive oxygen species, IR: ionizing radiation, UV: ultraviolet radiation.

REFERENCES

Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishida, Y. and Matsumoto, K. (1999) p38 mitogen-activated protein kinase can be involved in transforming growth factor β superfamily signal transduction in *Drosophila* wing morphogenesis. *Mol Cell Biol* **19**: 2322-2329.

Aggeli, I.K., Gaitanaki, C. and Beis, I. (2006) Involvement of JNKs and p38-MAPK/MSK1 pathways in H_2O_2-induced upregulation of heme oxygenase-1 mRNA in H9c2 cells. *Cell Signal* **18**: 1801-1812.

Akai, H. and Sato, S. (1971) An ultrastructural study of the haemopoietic organs of the silkworm, *Bombyx mori*. *J Insect Physiol* **17**: 1665-1676.

Akai, H. and Sato, S. (1973) Ultrastructure of the larval hemocytes of the silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae). *Inter J Insect Morphol Embryol* **2**: 207-231.

Altman, S.A., Randers, L. and Rao, G. (1993) Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol Prog* **9**: 671-674.

Altmann, F., Staudacher, E., Wilson, I.B. and März, L. (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj J* **16**: 787-791.

Andrewartha, H.G. (1952) Diapause in relation to the ecology of insects. *Biol Rev Camb Philos Soc* **27**: 50-107.

Arnold, J.W. and Hinks, C.F. (1976) Haemopoiesis in Lepidoptera. I. The multiplication of circulating haemocytes. *Can J Zool* **54**: 1003-1012.

Arnold, J.W. and Hinks, C.F. (1983) Haemopoiesis in Lepidoptera. III. A note on the multiplication of spherule cells and granular haemocytes. *Can J Zool* **61**: 275-277.

Ashburner, M. and Bonner, J.J. (1979) The induction of gene activity in *drosophilia* by heat shock. *Cell* **17**: 241-254.

Baluchamy, S. and Gopinathan, K.P. (2005) Characteraization of cyclin homolog from *Bombyx mori* nucleopolyhedrovirus. *Virus Res* **108**: 69-81.

Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. (1983) Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* **130**: 1910-1917.

Beaulaton, J. (1979) Hemocytes and hemocytopoiesis in silkworms. *Biochimie* **61**: 157-164.

Bulavin, D.V., Higashimoto, Y., Popoff, I.J., Gaarde, W.A., Basrur, V., Potapova, O., Appella, E. and Fornace, A.J., Jr. (2001) Initiation of a G_2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* **411**: 102-107.

Caraway, W.T. (1959) A stable starch substrate for the determination of amylase in serum

and other body fluids. *Am J Clin Pathol* **32**: 97-99.

Champlin, D.T. and Truman, J.W. (1998) Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. *Development* **125:**, 2009-2018.

Chapman, R.F. (1998) Thermal relations. In *The Insects Structure and Function*, pp 509-530. Cambridge: Cambridge University Press

Clotet, J., Escote, X., Adrover, M.A., Yaakov, G., Gari, E., Aldea, M., de Nadal, E. and Posas, F. (2006) Phosphorylation of Hsl1 by Hog1 leads to a G_2 arrest essential for cell survival at high osmolarity. *EMBO J* **25**: 2338-2346.

Courgeon, A.M., Becker, J., Maingourd, M., Maisonhaute, C. and Best-Belpomme, M. (1990) Early activation of heat shock genes in H_2O_2-treated *Drosophila* cells. *Free Radic Res Commun* **9**: 147-155.

Courgeon, A.M., Rollet, E., Becker, J., Maisonhaute, C. and Best-Belpomme, M. (1988) Hydrogen peroxide (H_2O_2) induces actin and some heat-shock proteins in *Drosophila* cells. *Eur J Biochem* **171**: 163-170.

Craig, C.R., Fink, J.L., Yagi, Y., Ip, Y.T. and Cagan, R.L. (2004) A *Drosophila* p38 orthologue is required for environmental stress responses. *EMBO Rep* **5**: 1058-1063.

Crossley, A.C. (1975) The cytophysiology of insect blood. *Adv Insect Physiol* **11**: 117-221.

Cymborowski, B. (2000) Temperature-dependent regulatory mechanism of larval development of the

wax moth (*Galleria mellonella*). *Acta Biochim Pol* **47**: 215-221.

Darzynkiewicz, Z., Bedner, E., Li, X., Gorczyca, W. and Melamed, M.R. (1999) Laser-scanning cytometry: A new instrumentation with many applications. *Exp Cell Res* **249**: 1-12.

Davidson, J.F., Whyte, B., Bissinger, P.H. and Schiestl, R.H. (1996) Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **93**: 5116-5121.

de Vries, H.I., Uyetake, L., Lemstra, W., Brunsting, J.F., Su, T.T., Kampinga, H.H. and Sibon, O.C. (2005) Grp/DChk1 is required for G_2-M checkpoint activation in *Drosophila* S2 cells, whereas Dmnk/DChk2 is dispensable. *J Cell Sci* **118**: 1833-1842.

Degols, G., Shiozaki, K. and Russell, P. (1996) Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. *Mol Cell Biol* **16**: 2870-2877.

Denlinger, D.L. (2002) Regulation of diapause. *Annu Rev Entomol* **47**: 93-122.

Dmitrieva, N.I., Michea, L.F., Rocha, G.M. and Burg, M.B. (2001) Cell cycle delay and apoptosis in response to osmotic stress. *Comp Biochem Physiol A Mol Integr Physiol* **130**: 411-420.

Doverskog, M., Bertram, E., Ljunggren, J., Ohman, L., Sennerstam, R. and Haggstrom, L. (2000) Cell cycle progression in serum-free cultures of Sf9 insect cells: modulation by conditioned medium factors and implications for proliferation and productivity. *Biotechnol Prog* **16**:

837-846.

Edgar, B.A. and Orr-Weaver, T.L. (2001) Endoreplication cell cycles: more for less. *Cell* **105**: 297-306.

Eguchi, D. and Iwabuchi, K. (2006) A new cell line from the wax moth *Galleria mellonella* Linne (Lepidoptera: Pyralididae). *In Vitro Cell Dev Biol Anim* **42**: 1-3.

Feir, D. (1979) Multiplication of hemocytes. In *Insect Hemocytes*. Gupta, A.P. (ed) pp 67-82. Cambridge: Cambridge University Press

Fertig, G., Kloppinger, M. and Miltenburger, H.G. (1990) Cell cycle kinetics of insect cell cultures compared to mammalian cell cultures. *Exp Cell Res* **189**: 208-212.

Fujiwara, Y., Shindome, C., Takeda, M. and Shiomi, K. (2006) The roles of ERK and p38 MAPK signaling cascades on embryonic diapause initiation and termination of the silkworm, *Bombyx mori*. *Insect Biochem Mol Biol* **36**: 47-53.

Gerbal, M., Fournier, P., Barry, P., Mariller, M., Odier, F., Devauchelle, G. and Duonor-Cerutti, M. (2000) Adaptation of an insect cell line of *Spodoptera frugiperda* to grow at 37 degrees C: characterization of an endodiploid clone. *In Vitro Cell Dev Biol Anim* **36**: 117-124.

Han, S.J., Choi, K.Y., Brey, P.T. and Lee, W.J. (1998) Molecular cloning and characterization of a *Drosophila* p38 mitogen-activated protein kinase. *J Biol Chem* **273**: 369-374.

Harari, P.M., Fuller, D.J. and Gerner, E.W. (1989) Heat shock stimulates polyamine oxidation by two

distinct mechanisms in mammalian cell cultures. *Int J Radiat Oncol Biol Phys* **16**: 451-457.

Hink, W.F. (1970) Established insect cell line from the cabbage looper, *Trichoplusia ni. Nature* **226**: 466-467.

Imanishi, S., Inoue, H., Kawarabata, T., Hara, K., Funakoshi, M., Yasunaga-Aoki, C. and Mitsuda, K. (2003) Establishment and characterization of a continuous cell line from pupal ovaries of Japanese oak silkworm *Antheraea yamamai Guerin-Meneville. In Vitro Cell Dev Biol Anim* **39**:1-3.

Jornot, L., Mirault, M.E. and Junod, A.F. (1991) Differential expression of hsp70 stress proteins in human endothelial cells exposed to heat shock and hydrogen peroxide. *Am J Respir Cell Mol Biol* **5**: 265-275.

Joy, O. and Gopinathan, K.P. (1995) Heat shock response in mulberry silkworm races with different thermotolerances. *J Biosci* **20**: 499-513.

Kaneko, H., Igarashi, K., Kataoka, K. and Miura, M. (2005) Heat shock induces phosphorylation of histone H2AX in mammalian cells. *Biochem Biophys Res Commun* **328**: 1101-1106.

Kani, S., Nakayama, E., Yoda, A., Onishi, N., Sougawa, N., Hazaka, Y., Umeda, T., Takeda, K., Ichijo, H., Hamada, Y. and Minami, Y. (2007) Chk2 kinase is required for methylglyoxal-induced G_2/M cell-cycle checkpoint arrest: implication of cell-cycle checkpoint regulation in diabetic oxidative stress signaling. *Genes Cells* **12**: 919-928.

Katsuma, S., Mita, K. and Shimada, T. (2007) ERK- and JNK-dependent signaling pathways contribute to *Bombyx mori* nucleopolyhedrovirus infection. *J Virol* **81**: 13700-13709.

Kim, I.S., Moon, H.Y., Yun, H.S. and Jin, I. (2006) Heat shock causes oxidative stress and induces a variety of cell rescue proteins in *Saccharomyces cerevisiae* KNU5377. *J Microbiol* **44**: 492-501.

Kim, Y.J., Lee, K.S., Kim, B.Y., Choo, Y.M., Sohn, H.D. and Jin, B.R. (2007) Thioredoxin from the silkworm, *Bombyx mori*: cDNA sequence, expression, and functional characterization. *Comp Biochem Physiol B Biochem Mol Biol* **147**: 574-581.

Koyama, T., Iwami, M. and Sakurai, S. (2004) Ecdysteroid control of cell cycle and cellular commitment in insect wing imaginal discs. *Mol Cell Endocrinol* **213**: 155-166.

Kuhl, N.M. and Rensing, L. (2000) Heat shock effects on cell cycle progression. *Cell Mol Life Sci* **57**: 450-463.

Kyriakis, J.M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* **81**: 807-869.

Lackie, A.M. (1988) Haemocyte behaviour. *Adv Insect Physiol* **21**: 85-177.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

LeBel, C.P., Ali, S.F. and Bondy, S.C. (1992) Deferoxamine inhibits methyl mercury-induced

increases in reactive oxygen species formation in rat brain. *Toxicol Appl Pharmacol* **112** : 161-165.

Lee, K.S., Kim, S.R., Park, N.S., Kim, I., Kang, P.D., Sohn, B.H., Choi, K.H., Kang, S.W., Je, Y.H., Lee, S.M., Sohn, H.D. and Jin, B.R. (2005) Characterization of a silkworm thioredoxin peroxidase that is induced by external temperature stimulus and viral infection. *Insect Biochem Mol Biol* **35**: 73-84.

Ling, E., Shirai, K., Kanekatsu, R. and Kiguchi, K. (2005) Hemocyte differentiation in the hematopoietic organs of the silkworm, *Bombyx mori*: prohemocytes have the function of phagocytosis. *Cell Tissue Res* **320**: 535-543.

Littleton, J.T. and Ganetzky, B. (2000) Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron* **26**: 35-43.

Lopez-Aviles, S., Grande, M., Gonzalez, M., Helgesen, A.L., Alemany, V., Sanchez-Piris, M., Bachs, O., Millar, J.B. and Aligue, R. (2005) Inactivation of the Cdc25 phosphatase by the stress-activated Srk1 kinase in fission yeast. *Mol Cell* **17**: 49-59.

Lopez-Girona, A., Furnari, B., Mondesert, O. and Russell, P. (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* **397**: 172-175.

Luther, E. and Kamentsky, L.A. (1996) Resolution of mitotic cells using laser scanning cytometry. *Cytometry* **23**: 272-278.

Lynn, D.E. (1995) A new insect cell line from the Colorado potato beetle. *In Vitro Cell Dev Biol Anim* **31**: 91-93.

Maeda, S. (1989) Gene transfer vectors of a baculovirus, *Bombyx mori* nuclear polyhedrosis virus, and their use for expression foreign genes in insect cells. *Invertebr Cell Syst Appl* **1**: 167-181.

Millar, J.B. (1999) Stress-activated MAP kinase (mitogen-activated protein kinase) pathways of budding and fission yeasts. *Biochem Soc Symp* **64**: 49-62.

Mita, K., Kasahara, M., Sasaki, S., Nagayasu, Y., Yamada, T., Kanamori, H., Namiki, N., Kitagawa, M., Yamashita, H., Yasukochi, Y., Kadono-Okuda, K., Yamamoto, K., Ajimura, M., Ravikumar, G., Shimomura, M., Nagamura, Y., Shin-I, T., Abe, H., Shimada, T., Morishita, S. and Sasaki, T. (2004) The genome sequence of silkworm, *Bombyx mori*. *DNA Res* **11**: 27-35.

Miyakoda, M., Suzuki, K., Kodama, S. and Watanabe, M. (2002) Activation of ATM and phosphorylation of p53 by heat shock. *Oncogene* **21**: 1090-1096.

Montell, C. (2003) The venerable inveterate invertebrate TRP channels. *Cell Calcium* **33**: 409-417.

Morgan, D.O. (1995) Principles of CDK regulation. *Nature* **374**: 131-134.

Mottier, V., Siaussat, D., Bozzolan, F., Auzoux-Bordenave, S., Porcheron, P. and Debernard, S. (2004) The 20-hydroxyecdysone-induced cellular arrest in G_2 phase is preceded by an

inhibition of cyclin expression. *Insect Biochem Mol Biol* **34**: 51-60.

Murhammer, D.W. (1991) Review and patents and literature. The use of insect cell cultures for recombinant protein synthesis: Engineering aspects. *Appl Biochem Biotechnol* **31**: 283-310.

Nagata, M., Seong, S.I. and Yoshitake, N. (1980) Variation of the hemolymph volume with larval development of the silkworm, *Bombyx mori*. *J Sericul Sci Japan* **49**: 453-454.

Nakagaki, M., Takei, R., Nagashima, E. and Yaginuma, T. (1991) Cell cycles in embryos of the silkworm, *Bombyx mori*: G_2-arrest at diapause stage. *Roux's Arch Dev Biol* **200**: 223-229.

Nakahara, Y., Kanamori, Y., Kiuchi, M. and Kamimura, M. (2003) *In vitro* studies of hematopoiesis in the silkworm: cell proliferation in and hemocyte discharge from the hematopoietic organ. *J Insect Physiol* **49**: 907-916.

Nebreda, A.R. and Porras, A. (2000) p38 MAP kinases: beyond the stress response. *Trends Biochem Sci* **25**: 257-260.

Nittono, Y. (1960). Studies on the blood cells in the silkworm, *Bombyx mori*, L. *Bull Sericul Exp Sta* **16**: 171-266.

Okazaki, T., Okudaira, N., Iwabuchi, K., Fugo, H. and Nagai, T. (2006) Apoptosis and adhesion of hemocytes during molting stage of silkworm, *Bombyx mori*. *Zool Sci* **23**: 299-304.

Ono, K. and Han, J. (2000) The p38 signal transduction pathway: activation and function. *Cell Signal* **12**: 1-13.

Palter, K.B., Watanabe, M., Stinson, L., Mahowald, A.P. and Craig, E.A. (1986) Expression and localization of *Drosophila melanogaster* hsp70 cognate proteins. *Mol Cell Biol* **6**: 1187-1203.

Pathak, J.P.N. (1986) Haemogram and its endocrine control in insects. In *Immunity in Invertebrates.* Brehelin M. (ed) pp 49-59. New York: Springer Verlag

Pearce, A.K. and Humphrey, T.C. (2001) Integrating stress-response and cell-cycle checkpoint pathways. *Trends Cell Biol* **11:** 426-433.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) Mitotic and G_2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**: 1501-1505.

Rafiee, P., Theriot, M.E., Nelson, V.M., Heidemann, J., Kanaa, Y., Horowitz, S.A., Rogaczewski, A., Johnson, C.P., Ali, I., Shaker, R. and Binion, D.G. (2006) Human esophageal microvascular endothelial cells respond to acidic pH stress by PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and Hsp27. *Am J Physiol Cell Physiol* **291**: C931-945.

Ratcliffe, N.A., Rowley, A.F., Fitzgerald, S.W. and Rhodes, C.P. (1985) Invertebrate immunity: basic concepts and recent advances. *Int Rev Cytol* **97**: 186-350.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S.J. (1997) Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage

to Cdk regulation through Cdc25. *Science* **277**: 1497-1501.

Schmidt-Nielsen, K. (1997) Temperature effects. In *Animal Physiology: Adaptation and Environment*. pp 218-239. Cambridge: Cambridge University Press

Siaussat, D., Mottier, V., Bozzolan, F., Porcheron, P. and Debernard, S. (2004) Synchronization of *Plodia interpunctella* lepidopteran cells and effects of 20-hydroxyecdysone. *Insect Mol Biol* **13**: 179-187.

Smith, A.V. and Orr-Weaver, T.L. (1991) The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development* **112**: 997-1008.

Smith, D.A., Toone, W.M., Chen, D., Bahler, J., Jones, N., Morgan, B.A. and Quinn, J. (2002) The Srk1 protein kinase is a target for the Sty1 stress-activated MAPK in fission yeast. *J Biol Chem* **277**: 33411-33421.

Sørensen, J.G., Nielsen, M.M., Kruhoffer, M., Justesen, J. and Loeschcke, V. (2005) Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress Chaperones* **10**: 312-328.

Sudo, M., Fujiwara, Y. and Horie, Y. (1999) Formulation of the relationship between rearing temperature and growth rate in 4th- and 5th-instar larvae of the silkworm, *Bombyx mori*, and their low development threshold temperature. *J Sericul Sci of Japan* **68**: 41-468. (in Japanese).

Takahashi, A., Matsumoto, H., Nagayama, K., Kitano, M., Hirose, S., Tanaka, H., Mori, E., Yamakawa, N., Yasumoto, J., Yuki, K., Ohnishi, K. and Ohnishi, T. (2004) Evidence for the involvement of double-strand breaks in heat-induced cell killing. *Cancer Res* **64**: 8839-8845.

Takamiya, K. and Nakajima, M. (1970) Effect of rearing temperature on the growth and development of the silkworm larvae fed on artificial diet. *Tech Bull Sericul Exp Sta* **84**: 1-12. (in Japanese).

Truman, J.W., Hiruma, K., Allee, J.P., Macwhinnie, S.G., Champlin, D.T. and Riddiford, L.M. (2006) Juvenile hormone is required to couple imaginal disc formation with nutrition in insects. *Science* **312**: 1385-1388.

Tu, Z.L., Kobayashi, Y., Kiguchi, K., Watanabe, H. and Yamamoto, K. (2002) Effects of heavy-ion radiosurgery on the hemopoietic function of the silkworm *Bombyx mori*. *J Radiat Res (Tokyo)* **43**: 269-275.

Uehara, T., Kaneko, M., Tanaka, S., Okuma, Y. and Nomura, Y. (1999) Possible involvement of p38 MAP kinase in HSP70 expression induced by hypoxia in rat primary astrocytes. *Brain Res* **823**: 226-230.

Volkov, R.A., Panchuk, II, Mullineaux, P.M. and Schoffl, F. (2006) Heat stress-induced H_2O_2 is required for effective expression of heat shock genes in *Arabidopsis*. *Plant Mol Biol* **61**

733-746.

Wago, H. (1991) Phagocytic recognition in *Bombyx mori*. In: *Immunology of Insects and other Arthropods*. Gupta, A.P. (ed) pp 215-235. Boca Raton: CRC Press

Wang, Y., Oberley, L.W. and Murhammer, D.W. (2001) Evidence of oxidative stress following the viral infection of two lepidopteran insect cell lines. *Free Radic Biol Med* **31**: 1448-1455.

Wigglesworth, V.B. (1972) Water and temperature. In *The Principles of Insect Physiology*. pp 663-699. New York: Halsted Press

Yamamoto, K., Banno, Y., Fujii, H., Miake, F., Kashige, N. and Aso, Y. (2005) Catalase from the silkworm, *Bombyx mori*: gene sequence, distribution, and overexpression. *Insect Biochem Mol Biol* **35**: 277-283.

Yamashita, M. and Iwabuchi, K. (2001) *Bombyx mori* prohemocyte division and differentiation in individual microcultures. *J Insect Physiol* **47**: 325-331.

Zhang, Z., Leonard, S.S., Huang, C., Vallyathan, V., Castranova, V. and Shi, X. (2003) Role of reactive oxygen species and MAPKs in vanadate-induced G_2/M phase arrest. *Free Radic Biol Med* **34**: 1333-1342.

Zhou, J., An, H., Xu, H., Liu, S. and Cao, X. (2005) Heat shock up-regulates expression of Toll-like receptor-2 and Toll-like receptor-4 in human monocytes via p38 kinase signal pathway. *Immunology* **114**: 522-530.

Zhou, M., Zhang, A., Lin, B., Liu, J. and Xu, L.X. (2007) Study of heat shock response of human umbilical vein endothelial cells (HUVECs) using cDNA microarray. *Int J Hyperthermia* **23**: 225-258.

Zhuang, Z.H., Zhou, Y., Yu, M.C., Silverman, N. and Ge, B.X. (2006) Regulation of *Drosophila* p38 activation by specific MAP2 kinase and MAP3 kinase in response to different stimuli. *Cell Signal* **18**: 441-448.